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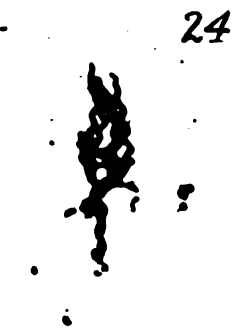
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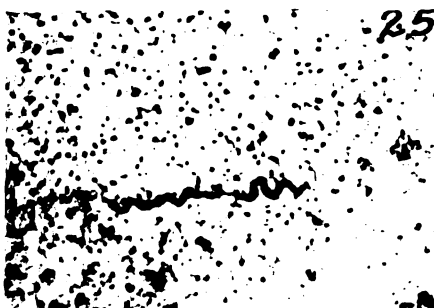
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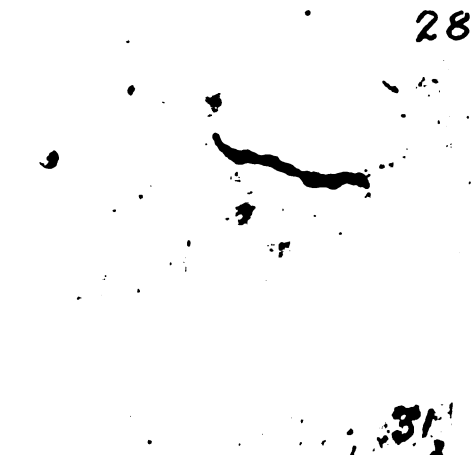
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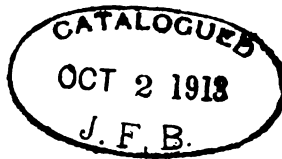
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STUDIES
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UEBER DIE VITALE FÄRBUNG DES TUBERKELS.*

VON DR. BOWMAN, WINTERNITZ, UND EVANS.

(Aus dem anatomischen Laboratorium der Johns Hopkins University,
Baltimore.)

In einer vorläufigen Mitteilung gebe ich hiermit das Resultat einer langen Reihe von Experimenten, die ich in Verbindung mit Dr. F. B. Bowman und Dr. Milton Winternitz in der Johns Hopkins-Universität gemacht habe. Goldmann¹⁾ hat jüngst über Versuche berichtet, die er bei weissen Mäusen mit intraperitonealer Injektion von Bacillen der Hühner- und Rindertuberkulose unter Anwendung der vitalen Färbung gemacht hat. Es ist ihm die vitale Färbung von Tuberkeln in der Leber und der Milz nach der Infektion mit Bacillen der Hühnertuberkulose gelungen. Unsere Versuche sind von denen Goldmanns insofern verschieden, als wir bei Kaninchen isolierte Tuberkel der Leber durch die Injektion der Bacillen der Rindertuberkulose in die Vena colica erzeugt haben.

Gleichzeitig mit der Injektion der Bacillen wurde Trypanblaulösung in den Kreislauf gebracht, und zwar dadurch, dass 20 ccm einer 1-proz. Lösung in die Ohrvene gespritzt wurden.

Wir haben nun sorgfältigst alle Veränderungen in der Leber verfolgt, indem wir die Tiere in Zwischenräumen von einer halben Stunde, von 6, 24, 36, 40, 48, 54, 72 und 96 Stunden nach der Bacilleninjektion getötet haben. Unsere ausführliche Arbeit soll demnächst in the *Journal of Experimental Medicine* publiziert werden.

Wir geben hier nur unsere hauptsächlichsten Befunde. Unsere Untersuchungen haben ergeben, dass die Riesenzellen bei frischer Tuberkulose endothelialen Ursprungs sind und dass dieselben eine

* Mit Unterstützung des Rockefeller Institute for Medical Research, New York.

1) Ueber die innere und äussere Sekretion des gesunden und kranken Organismus im Lichte der vitalen Färbung. (Bruns, Beiträge. Bd. 78. 1912.)

ausgesprochene Elektivität für den vitalen Farbstoff besitzen. Wenn man die Leber kurz nach der Bacilleninjektion ($\frac{1}{2}$ bis 6 Stunden) untersucht, so findet man die Bacillen vorwiegend in grösseren Haufen mitten in Thromben portaler Venenäste. Weisse Blutzellen, in Form multinukleärer, später mononukleärer Leukocyten haben sich angesammelt und tragen zur Bildung der Thromben bei, ohne aber in hervorragender Weise sich an der Phagocytose der Bacillen zu beteiligen. Die Bacillen, welche allmählich anfangen, sich zu vermehren, werden sehr bald in grösserer Menge im Körper der endothelialen (Kupfferschen) Zellen gefunden. Die Anwesenheit der Bacillen ruft in solchen Zellen eine deutliche Schädigung des Protoplasmas hervor, welche sich durch eine diffuse vitale Blaufärbung des Cytoplasmas kund gibt, die niemals in der normalen Zelle gefunden wird. Andererseits beobachtet man in der Nachbarschaft des Thrombus endotheliale Zellen in allen Stadien der Mitose. Ob auch eine Amitose vorkommt, ist nicht ausgeschlossen. Sei dem wie ihm wolle, doppelkernige und mehrkernige intravaskuläre Endothelzellen (Riesenzellen) werden gebildet, welche alle vital gefärbt sind. Wenn diese mehrkernigen, vital gefärbten Zellmassen anfangs auch wenig Bacillen enthalten, so nimmt doch die Zahl der Bacillen innerhalb derselben bald zu, so dass man bei Anwendung der spezifischen Färbung neben roten Stäbchen vital gefärbte Granula im Protoplasma nachweisen kann. Die Blaufärbung dieser Riesenzellen ist eine so ausgesprochene, dass dieselbe sich schon bei schwacher Vergrösserung scharf von dem übrigen Lebergewebe abhebt. Es kann gar keinem Zweifel unterliegen, dass die Tuberkelbacillen diese prägnante Elektivität des Zellplasmas für den vitalen Farbstoff hervorgerufen haben. Die Elektivität der tuberkulösen Riesenzellen für den vitalen Farbstoff lässt sich noch in einer anderen Weise schön demonstrieren. Normale Tiere werden zuerst mit einem roten vitalen Farbstoff (Diamin-Scharlach) behandelt, wonach die Leber und Kupferzellen rot gefärbte Granula enthalten. Das Versuchstier wird dann intravenös mit einer Bacillenkultur und kurz darauf mit einer schwachen Lösung eines blauen vitalen Farbstoffes (Trypanblau) injiziert. Hiernach erscheinen die tuberkulösen Riesenzellen vital blau gefärbt, wobei sie sich scharf von ihrer roten Umgebung abheben.

Die mitgeteilten Tatsachen deuten darauf hin,

1) eine wie wichtige Rolle Endothelzellen bei hämatogenen Infektionsprozessen im allgemeinen, bei der Tuberkulose im besonderen spielen.

2) Wie gross die Elektivität der tuberkulösen Riesenzellen für vitale Farbstoffe ist.

3) Von welcher Bedeutung für chemotherapeutische Bestrebungen die Untersuchungen mit vitalen Farbstoffen sind.

EXPERIMENTAL POLIOMYELITIS IN MONKEYS.

THIRTEENTH NOTE: SURVIVAL OF THE POLIOMYELITIC VIRUS IN THE STOMACH AND INTESTINE.

By SIMON FLEXNER, M.D., PAUL F. CLARK, Ph.D., AND
A. R. DOCHEZ, M.D.

(From The Rockefeller Institute for Medical Research, New York.)

The view of the nasopharyngeal site of entrance into and exit from the infected body of the poliomyelitic virus has steadily gained support from experiments on monkeys¹ and from observations on cases of epidemic poliomyelitis in man. The recent observations of Kling, Wernstedt and Pettersson² of Stockholm have an especially important bearing on this subject. These observers have demonstrated the virus by inoculations made into monkeys, in the mucus contained in washings from the nose and mouth of acute examples of poliomyelitis both during life, in patients who recovered, and after death in individuals who succumbed. The virus had previously been detected by inoculation tests in the nasal³ and buccal mucosa and in the tonsils^{3, 4} in fatal human cases of the disease. The findings of Kling, Wernstedt and Pettersson would appear not only to complete the chain of evidence in support of the nasopharyngeal route of infection in man, but to add still another important confirmation to the prevailing views regarding the sources of infection in poliomyelitis, in that they establish the presence of the virus in the nasal and buccal cavities in cases of poliomyelitis

1. Flexner and Lewis: THE JOURNAL A. M. A., Feb. 12, 1910, p. 535; Jour. Exper. Med., 1910, xii, 227. Flexner: THE JOURNAL A. M. A. Sept. 24, 1910, p. 1105.

2. Kling, Wernstedt and Pettersson: Ztschr. f. Immunitätsforsch. u. exper. Therap. Orig., 1912, xii, 316, 657.

3. Flexner and Clark: THE JOURNAL A. M. A. Nov. 18, 1911, p. 1685.

4. Landsteiner, Levaditi and Pastia: Semaine méd., 1911, No. 25, p. 296.

of the meningitic and abortive types in which frank paralysis has not occurred.

But these authors have observed still another situation in which the virus occurs with great constancy, namely, in the intestine, both in the mucus contained within the small intestine, as discovered in fatal cases, and in the washings from the large intestine as determined in patients during life. The regular occurrence of the virus in these situations in human cases of poliomyelitis immediately raises the question of the manner of its entrance into the intestines. Since the virus has now been shown to exist in the nasal and buccal mucous membranes and in their mucus secretions it is presumable that it reaches the gastro-intestinal tract with the swallowed secretions. In this case the virus must be capable of surviving in the stomach and passing out alive, and of resisting the action of the intestinal ferments and bacterial flora. The capacity of the virus to survive in the stomach and small intestine was tested in the following manner.

REPORT OF EXPERIMENT.

A milky suspension of the spinal cord of a recently paralyzed poliomyelitic monkey was prepared of which 50 c.c. were introduced by stomach tube into a *rhesus* monkey that had not been fed for fifteen hours. After the lapse of two hours the animal was deeply etherized and the stomach and about 20 cm. of the duodenum and jejunum were enclosed separately between ligatures. The ether was then increased and the monkey killed. The two organs were removed and their surfaces washed in sterile saline solution. They were then opened. The stomach was empty except for a quantity of mucus at the pyloric extremity. The intestine was empty. The surfaces of the mucosæ were washed in 50 c.c. of sterile saline solution and the washings collected separately. After agitation each was filtered under pressure through Berkefeld candles. The filtrates were sterile. Four c.c. of each filtrate were inoculated intracerebrally into the *Macacus rhesus* monkeys. The two inoculated monkeys presented the first symptoms of poliomyelitis on the fourth and fifth days, respectively. The animal injected with the filtrate prepared from the intestinal mucus showed symptoms one day earlier than the animal injected with the filtrate prepared from the contents of the stomach. The progress of the disease was similar in the two animals. The muscles of the extremities became weak and those of the trunk paralyzed. Monkey A was etherized on the sixth day and the autopsy performed at once. Monkey B died suddenly on the sixth day from respiratory paralysis. The spinal cord and medulla of both animals showed characteristic lesions of poliomyelitis.

CONCLUSIONS.

The deduction from these experiments is obvious. Since the poliomyelitic virus occurs in the nasal and buccal mucus in human cases of poliomyelitis it is inevitably taken into the stomach with the swallowed saliva. The virus survives the action of both the gastric and intestinal secretions and persists for a time in these organs. In human beings it leaves the body, in part, with the intestinal discharges, which are therefore a potential source of infection. It remains to be determined whether in the monkey artificially fed with the virus it also passes out in a viable state with the dejecta.

SOME OBSERVATIONS OF THE THORACIC DUCT LYMPH AFTER INJECTION OF OIL OF TUR- PENTINE INTO THE PERITONEAL CAVITY OF THE DOG.*

By ROBERT L. DIXON, M.D.

*(From the Pathological Laboratory of the University of Michigan,
Ann Arbor.)*

The physical relation existing between serous cavities and the vessels of the lymphatic circulation has been studied in the past by many investigators, and efforts have been made to trace the paths taken by foreign material, soluble and insoluble, in leaving the abdominal cavity.

In this study we desired, particularly, to know if leucocytes that have entered the serous cavity as the result of an inflammatory process are returned to the circulation, and, if so, by what route. Some notice was also taken of the disposition made of insoluble pigment granules introduced into the abdominal cavity. In these experiments healthy dogs were used.

In order to establish the process of inflammation in the serous cavity, five to ten cubic centimeters of oil of turpentine were injected under morphin anesthesia through the abdominal wall by means of an ordinary syringe. In several instances a red dye was conveyed along with the turpentine into the abdominal cavity. At different intervals after the injection, the animals were examined.

Under anesthesia (chloretone) the thoracic duct was isolated in the neck, and a cannula introduced. Total and differential counts of the cells in the thoracic duct lymph were made in the usual way. The abdominal cavity was opened under anesthesia, care being taken to prevent blood from mixing with the fluid collected in the peritoneal cavity. Counts and study of types of cell in the effusion were then made. The observations and conclusions based upon this set of experiments are briefly stated in this paper.

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, April 15, 1912.

The amount and nature of the fluid found varied greatly with different intervals. While the changes as determined in different dogs were not identical, yet there existed a general relationship between the factors involved.

The *first or earliest effect* noted was a marked congestion of the vessels of the serous coat, this being accompanied by an effusion. The effusion had a very pale yellowish color, was of a watery consistency, and contained very few cells. These cells were of the small mononuclear type. After four to six hours the fluid clotted in a test-tube, forming a very soft, jelly-like mass.

It was not possible to determine the absolute number of cells per cubic millimeter of this fluid because of the fact that as the animals lay for some time in a given position the cells settled, and no particular quantity truly represented the total. Nevertheless, from the counts made of the cells in this early fluid the number was probably not more than two to three hundred per cubic millimeter.

Four to six hours after the injection of the turpentine, the cover-glass smears showed very few cells. Practically without exception these were the small lymphocytes and showed no marked variation in size. Red blood cells were absent. The fluid present was about fifty cubic centimeters.

At the end of the *second day* the effusion had increased to two hundred cubic centimeters. The color was more nearly pink, due to the increased number of red blood cells. The density of the fluid was greater, and it clotted in one to two hours into a more compact mass. The calculated number of the white cells per cubic millimeter was about ten thousand.

These cells varied greatly in many respects. The majority were mononuclears. Most of these were small lymphocytes and some were very small indeed, but many were unusually large, being much larger than the cells ordinarily found in the lymph or blood. Of these very large mononuclear cells there were about a dozen to a cover-glass. In diameter these cells were approximately thirty to forty microns. They had a large, compact, blue-staining nucleus with a peripheral band of protoplasm stained bright pink. These were probably mesothelial cells.

All the cells showed the effects of soaking in the fluid. This was

indicated especially by their frayed and indefinite outlines. Many of the cells were phagocytes and contained red blood cells, pigment, and cell detritus. Red blood cells were very numerous.

At the end of the *third day* the effusion amounted to two to four hundred cubic centimeters. The density and the cellular content were greater than on the second day and within half an hour the fluid clotted into a rather firm mass. It was of a light creamy color and so stringy as to make it difficult to make thin cover-glass smears. The smears showed a relative increase in the number of polymorphonuclear cells. There were many mononuclear elements showing much disintegration, and a great amount of a stringy fibrinous substance. The number of red cells was much decreased.

On the *fourth day* the amount of the fluid was much less, of a thicker, more creamy consistency, and clotted into a firm mass within half an hour. There was now a definite preponderance of polymorphonuclear cells. There were also, however, many large mononuclear cells, usually with loosely arranged, palely stained nuclei. Red cells were practically absent.

On the *fifth day* the fluid had practically disappeared, and all that remained was a layer of fibrin. This was on the peritoneum, but especially on the diaphragm and on the surface of the liver and spleen. In the meshes of this fibrin were many pigment granules either free or enclosed within phagocytes.

Evidently it is not possible to compare the output from the thoracic duct in these instances with what it was before the injections were made. But from a knowledge of the findings in a great many cases it is possible to assume that certain types of cells were present in certain relative proportions, and to consider wide variations from this standard as unusual and perhaps as a consequence of certain factors and agencies employed in the instance in question.

The most extensive and detailed enumeration of the cell content of the lymph from the thoracic duct has been reported from this laboratory by Rous.¹ His report is based upon a careful consideration of the number and types of cells found in the lymph from twenty-three dogs in which, as far as could be ascertained, no complicating conditions existed.

¹ Rous, Peyton, *Jour. Exper. Med.*, 1908, x, 537.

My counts of the cells of the lymph can well be compared with the standard established by the findings of Rous. The observations of Delamere, and of Biedl and Decastello, as well as my own in a previous set of experiments, are also taken into account in this connection. Rous² states: "Mast cells are not a constituent of the lymph of the normal dog. Polymorphonuclear neutrophils are only present as a result of blood admixture. Lymphocytes, by which are meant non-granular cells with a round or oval nucleus smaller than an entire polymorphonuclear neutrophil from the same animal, form an average of 87.6 per cent. of the cells." The percentage goes as low as 69.8 per cent. and as high as 96.8 per cent. "Typical transitional forms are rare in the lymph. . . . Large mononuclear cells—non-granular mononuclear elements larger than the lymphocyte as above defined—average 5.2 per cent. of the lymph's leucocytes." From the differences in the cell counts in individual dogs it is evident that in normal animals great variations from these average percentages occur. Since the variations are normally so great, the proportions of the cells must be altered extremely before we can be sure that a given agency has produced a noteworthy effect.

I have made a total and differential count of the cells in the lymph of the thoracic duct in each of my cases, the results of which are given in table I. By comparing the data in this table with those already cited, we may draw our conclusions.

If such a table could be made to apply to a single animal over a period of five days, and the test repeated on as many animals as are here represented, it would be very easy to form rather definite conclusions as to the types of the cells and the degree of variation in the cell formula of the lymph under varying conditions and agencies. Such procedures, however, do not seem to be practicable.

We can do little more than compare the relative proportions of the various elements in different animals and the approximate rates of flow.

The *small mononuclear* cells, which primarily constitute the bulk of cells in the lymph, here show a range of from 63.8 per cent. to 92.4 per cent. These extremes cannot be said to be far from the

² Rous, Peyton, *loc. cit.*

TABLE I.

Total and Differential Counts of Cells in the Thoracic Duct at Varying Intervals after Injection of Oil of Turpentine into the Peritoneal Cavity of Dogs. Each Set of Counts Represents a Different Animal. Eleven Animals Were Used.

After injection.	Small mono-nuclears.		Large mono-nuclears.		Polymorpho-nuclear neutrophils.		Eosinophils.		Transi-tionals.		Total counted.	Total per c.mm.	Flow in 10 min. in c.c.
	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.	No. of cells.	Per cent.			
4 hrs.	476	84.5	59	10.4	7	1.2	9	1.6	12	2.1	563	8,650	7.0
6 hrs.	403	79.6	77	15.2	6	1.1	3	0.56	16	3.1	505	5,500	12.5
36 hrs.	417	73.5	105	18.5	12	2.1	7	1.2	26	4.5	567	9,975	9.5
2d day	348	71.6	91	18.7	27	5.5	2	0.4	18	3.7	486	7,500	14.0
2d day	240	63.8	73	19.4	19	5.0	12	3.2	32	8.5	376	10,400	7.0
3d day	427	73.7	96	16.6	7	1.2	6	1.0	42	7.2	578	9,225	13.0
3d day	318	70.4	85	18.8	21	4.6	4	0.9	23	5.0	451	12,600	13.5
4th day	409	83.4	28	5.7	26	5.3	18	3.7	9	1.8	490	11,000	8.0
4th day	381	89.4	19	4.4	14	3.3	3	0.7	9	2.1	426	19,100	11.0
5th day	412	91.9	15	3.3	5	1.1	11	2.4	5	1.1	448	6,475	7.5
5th day	341	92.4	13	3.5	9	2.4	2	0.54	4	1.09	396	11,500	5.0

percentages taken as a standard, although the lowest count is below the minimum percentage obtained by Rous.

The variations of percentage with reference to the effusion are significant. At first when the amount of effusion was small and the cells contained in it were few, the percentage of mononuclear cells in the lymph was relatively high. Later, as the *effusion* became characterized by mononuclear cells of this type, the percentage in the *lymph* diminished. This is, perhaps, not what we should expect if we were to take the view that the cells of the effusion are supplied from the blood, and that the supply in the blood is maintained by the cells coming from the thoracic duct. But even from this point of view it seems possible to explain the findings.

The first effect of the injection of the turpentine is a demand upon the protective forces of the body. This is evidenced by the prompt pouring out of a fluid that acts as a diluent and perhaps as an antagonist chemically to the irritant agent. Simultaneously with this there is an increased activity on the part of the tissues or organs which furnish the cellular elements. Hence a high percentage of the elements in the lymph at this time consists of cells that are about to be passed into the blood and later into the peritoneal cavity. But

there is no doubt that many of the cells go to the seat of activity without passing through the ordinary channels. This is especially true later in the process, and accounts for the continued relative increase in mononuclear cells in the effusion after the relative decrease in the thoracic duct. Later in the process the percentage of the small mononuclear cells in the lymph rises again to a high mark, due perhaps to the reverting of the supply into the usual channel and to the taking up of many of these cells from the peritoneal cavity. That the mononuclear cells are taken up from the peritoneal cavity is evidenced by the great numbers of poorly stained, loosely formed, and misshaped cells of this type that are seen in the smears from the lymph at this time.

The *large mononuclears* maintain a ratio that is practically the reciprocal of that of the small mononuclears. The percentage of the large mononuclear cells in the lymph is high immediately before and during the time when their number in the peritoneal fluid is greatest. Late in the process, however, the ratio of the large mononuclear cells in the lymph to those in the peritoneal fluid is relatively low.

This may indicate that the supply of the large mononuclears is not immediately restored or that these cells do not return to the thoracic duct once they have been passed into the serous cavity. However, the number of these cells that show the effects of soaking in the fluid, as evidenced by loss of definite outline, loose chromatin, and pale staining, would seem to indicate that many of them are returned to the thoracic duct. Although the percentages of the large mononuclear cells in the lymph in my animals varied greatly, they did not go beyond the limits found in normal conditions.

Polymorphonuclear neutrophils constituted from 1.1 per cent. to 5.5 per cent. in this series of counts. This ratio is higher than the standard adopted, although Rous shows one instance of 11 per cent. with apparently only a slight admixture of blood. However, in my animals the number of polymorphonuclears apparently bears no relation to the other features of the condition. It would seem, then, that whatever may be the number of this type of cells in the lymph of the thoracic duct, it is not determined by the number furnished to the blood, or by the number removed from an effusion

containing a large number of these cells. This may be taken as another indication that the thoracic duct is not a highway for the polymorphonuclear neutrophils.

The relation of the *eosinophils* to the process under study appeared to be no greater than that of the polymorphonuclear cells just considered. My ratios ranged from 0.4 to 3.7 per cent.

The number of *transitional cells* in the lymph of my animals was much greater than was anticipated in view of what has been stated regarding their presence in the lymph of normal dogs. Their numbers range from 1.09 to 8.5 per cent. It may perhaps be significant that in the lymph the increase precedes and accompanies the increase of the polymorphonuclear cells in the effusion. However, the percentage of the transitional cells in the lymph is too low for us to attribute the supply of these cells in the blood to the number received from the thoracic duct. The small number of the cells in the lymph may be merely indicative of the way in which the body draws upon all possible sources of supply in conditions such as have been induced here.

The total cell count per cubic millimeter is variable and in itself does not bear a definite relation to the process. If at times during the course of the process we could compare each case with itself before the process began, perhaps more significant factors would appear. The rate of flow was as variable and as inconsistent, apparently, as the total cell count.

In considering all of these points it must be remembered that many factors, which it is difficult to have exactly alike in each case, have a bearing upon the results. The size and activity of the animal, the nature of the food, and the time it is taken are some of these factors.

In addition to the experiments just reported, others were made. One was suggested by a report of Opie³ in which in dogs an effusion into one pleural cavity was secured by injecting turpentine into the other. This result was obtained only occasionally. To ascertain if the site giving rise to the effusion is identical with the field of irritation, I opened the peritoneal cavity of a dog, under chlorotone anesthesia, and holding the intestines upward toward the diaphragm,

³ Opie, E. L., *Jour. Exper. Med.*, 1907, ix, 391.

I placed a piece of gauze saturated with turpentine in the right inguinal region. Gauze was used rather than the free turpentine so as to prevent, as much as possible, any spread of the irritant. Holding the irritant in this position a quantity of fluid (fifteen to twenty cubic centimeters) collected within an hour, and it was plainly evident that portions of the serous membrane far distant were pouring out the fluid as well as the field locally irritated. This indicates that some nervous mechanism probably takes part in the process, and this explanation may also hold for the observations of Opie relative to the two pleural cavities.

The disposition of pigment placed within the peritoneal cavity was also considered in this connection. From the extensive investigation by Starling and Tubbey⁴ it is concluded that soluble coloring matters are absorbed to the greatest degree from the serous cavity directly into the blood-vessels, the urine showing the color earlier than the lymph in the thoracic duct.

The method by which insoluble pigment is disposed of, and the route the pigment takes have also received much attention. The method established by von Recklinhausen⁵ has been modified by more recent observers so that now we may consider that there are at least two general ways by which pigment is removed; namely, (1) through the agency of phagocytes, and (2) by the mechanical aspiration of the substance into the lymph channel by the respiratory movements. This subject, based upon the histology of the tissues concerned, is completely discussed by MacCallum.⁶

In my experiments I desired to see if the phagocytic cells followed the channels of the thoracic duct, but I failed to find a single polymorphonuclear cell containing pigment in the lymph from the thoracic duct. An occasional mononuclear enclosed a few granules of the red pigment previously injected into the cavity. However, pigment in appreciable quantities was found in the retroperitoneal and mediastinal glands. This would indicate that the phagocytes take a shorter and more direct route in depositing the foreign substance in the glands.

⁴Starling, E. H., and Tubbey, A. H., *Jour. Physiol.*, 1894, xvi, 140.

⁵von Recklinhausen, F., *Virchows Arch. f. path. Anat.*, 1863, xxvi, 172.

⁶MacCallum, W. G., *Bull. Johns Hopkins Hosp.*, 1903, xiv, 105.

CONCLUSIONS.

Injection of oil of turpentine into the peritoneal cavity of a dog calls forth immediately an exudate of fluid from the surrounding tissues. The amount of fluid reaches the maximum on the third day and has practically disappeared on the fifth day.

The cell content of this fluid is very small at first, but increases rapidly. The type of the predominating cells also changes. In the early exudate the small mononuclear cells are numerous and the large mononuclears few. Later the number of large mononuclear cells is increased and ultimately the polymorphonuclear cells preponderate. Various forms of atypical cells also occur.

Much of the fluid and many of the cells are removed by way of the thoracic duct. The counts of the cells in the thoracic duct and the estimates based on these indicate that the duct does not remove all of the fluid or cells from the peritoneal cavity. Much fluid is probably taken back directly into the blood, as are many of the cells. Some of the cells make their way to the lymph nodes, while many perhaps undergo complete autolysis in the serous cavity.

The polymorphonuclear cells do not enter the thoracic duct in great numbers. Examination of the lymph from the thoracic duct in the case of my dogs showed the types of cells that are usually found there.

The variation in small mononuclear cells is so related to the cell content of the peritoneal effusion as to indicate that the supply in the blood is maintained from this source. The form and staining qualities of the cells indicate that many of the small mononuclear cells are returned to the thoracic duct.

The ratio of polymorphonuclear cells present in the lymph bears no definite relation to the other features of the process. The transitional cells were increased in number and in their ratio to other cells. The increase in the transitional types accompanies an increase in large mononuclear cells and a decrease in small mononuclears.

None of the atypical forms of cells found in the effusion were seen in the lymph.

The number of eosinophils is without apparent relation to the other features of the process.

No polymorphonuclear cells containing the pigment injected were found in the lymph of the thoracic duct, and the number of mononuclear cells containing pigment was small. Much pigment was deposited in the lymph nodes. Detailed and definite conclusions as to the relation between the cells of the lymph and those of the effusion cannot be arrived at satisfactorily without repeated observations on the same animal.

Dr. Warthin examined many of the smears from these cases and frequently controlled the conclusions regarding the various types of cells. Dr. P. F. Morse assisted me frequently with the operative procedures and with the routine counting of the cells.

TREPONEMA MUCOSUM (NEW SPECIES), A MUCIN-
PRODUCING SPIROCHÆTA FROM PYORRHEA
ALVEOLARIS, GROWN IN PURE CULTURE.*

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATE 18.

Pyorrhea alveolaris is generally regarded as the sequel to a pathological metabolism known as the uric acid diathesis. Whatever the primary predisposing factors may be, the characteristic fetid odor from the affected mouth betrays the presence of certain putrefactive microorganisms in the perialveolar tissues which surround the loosened roots of the diseased teeth. The bacteriological studies which, on account of the lack of cultural methods for most of the organisms concerned, were necessarily limited to their morphology, helped but little, for the varieties of the microbes therein found do not differ greatly morphologically from those commonly seen in the gingival deposit on unclean teeth of individuals who do not have pyorrhea alveolaris. Most authorities mention the presence of *Spirochæta buccalis*, *Treponema macrodentium*, *Treponema microdentium*, fusiform bacilli, and, occasionally, a spirillum allied to that of Vincent. But which of these organisms is most responsible for the fetid odor could not be determined for the reason that until very recently neither the spirochætæ nor the other forms were isolated in pure cultures.

The fusiform bacilli and spirilla of Vincent's type have been obtained in pure culture by Ellermann,¹ Weaver and Tunncliff,² Mühlens,³ and others,⁴ who found that these organisms produce the peculiar fetid odor characteristic of pyorrhea alveolaris.

* Received for publication, May 15, 1912.

¹ Ellermann, V., *Centralbl. f. Bacteriol., Orig.*, 1904, xxxvii, 729.

² Weaver, G. H., and Tunncliff, R., *Jour. Infect. Dis.*, 1905, ii, 446.

³ Mühlens, P., *Deutsch. med. Wchnschr.*, 1906, xxxii, 797.

⁴ Tunncliff, R., *Jour. Infect. Dis.*, 1906, iii, 148.

Of the spirochætæ, one medium type, *Treponema macrodentium*, and one small type, *Treponema microdentium*, have been cultivated by me from non-pyorrheal cases,⁵ but the macrodentium produces no odor and the microdentium produces a putrefactive odor which is somewhat different in nature from that which comes from pyorrhea alveolaris.

In the present communication I shall describe a small spirochæta which was isolated from the pus derived from a case of pyorrhea alveolaris. Morphologically the organism resembles greatly *Treponema pallidum* and *Treponema microdentium*, but it differs from both in several principal biological properties. Its most striking feature is its capacity to produce in pure culture a mucin and a strong fetid odor. Since this species has, for the first time, been recognized and isolated from other varieties, I propose for it the name *Treponema mucosum*. At present I shall not discuss the question of how much this particular spirochæta contributes towards the production of the fetid odor in the pyorrheal discharge, but I shall describe the method of cultivation and its morphology and biology in pure culture.

Method of Cultivation.—The purulent discharge of pyorrheal teeth is collected by means of a sterile capillary pipette and then suspended in a few cubic centimeters of sterile citrate solution. By means of a capillary pipette this emulsion is inoculated into a number of tubes of solid culture media consisting of one part of ascitic fluid, two parts of ordinary agar, and having a piece of sterile fresh rabbit kidney at the bottom; the whole medium is covered with a layer of sterile paraffin oil. The technique is the same as that used for obtaining the pallidum or refringens from a contaminated chancre or condyloma. After inoculation the tubes are incubated at 37° C. for about ten days. The whitish semi-transparent growth may be seen to spread out from the stab canal which is now filled with a dense growth of various bacteria. The tube is cut open at the middle, the freshly exposed surface of the agar column is carefully sterilized with a sublimate alcohol solution, the moisture carefully wiped off and then the cloudy colony of the spirochætæ is reached by inserting a sterile capillary pipette through

⁵ Noguchi, H., *Jour. Exper. Med.*, 1912, xv, 81.

the clean surface. A small portion of the colony is thus taken out and transferred to a new medium. By repeating this process of purification one finally obtains a pure culture.

**Properties of the Pure Cultures.*—In the ascitic agar medium, containing fresh sterile tissue, the colonies become visible within twenty-four to forty-eight hours, and with the exception of the upper 1.5 centimeters the entire agar column is rendered unevenly opalescent. The individual colonies are recognized as dense, whitish turbidities of varying sizes concentrically arranged but having no definite outer boundaries. In a rapidly growing culture the neighboring colonies unite with each other by their gradually thinning peripheries, thus making the whole tube quite opalescent, although the original colonies still stand out more or less distinctly.

No gas is formed. The culture gives off a strong fetid odor that is easily recognized at a distance. After growing for two or three weeks the tissue in the culture becomes at first greyish and then quite dark (similar changes were found in the microdentium culture). The culture now contains a varying amount of mucin. When the media is broken up this is seen as an extremely fine string stretching from one piece of agar to another. The mucin string is not sticky, but draws out as a fine filament. I have seen no bacteria which produce a similar mucin.

The organisms remain alive for several weeks in the same culture at 37° C. The mucin-producing faculty has apparently suffered through repeated transplantations and at present, in its sixteenth generation within 160 days, the culture has almost lost this quality. On the other hand, its capacity to produce a bad odor has in no wise diminished.

Like other treponemata, the mucosum is an anaerobe and requires the presence of serum constituents for its growth. It grows well without the presence of fresh tissue. The fluid medium is made markedly opalescent without forming a definite coagulum. In this fluid medium the growth produces a very strong odor.

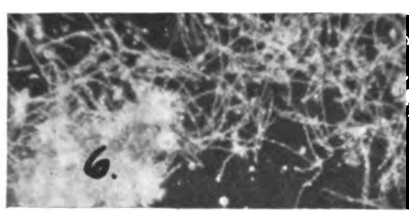
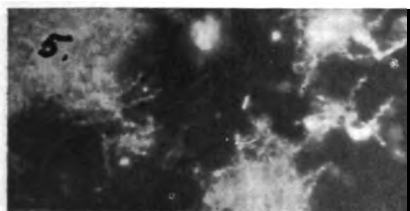
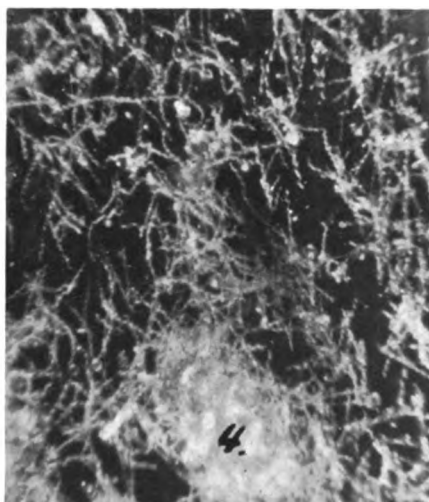
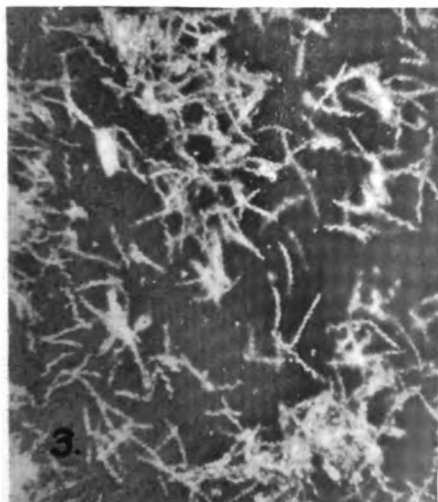
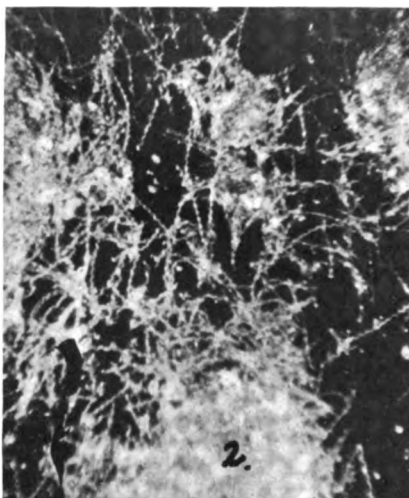
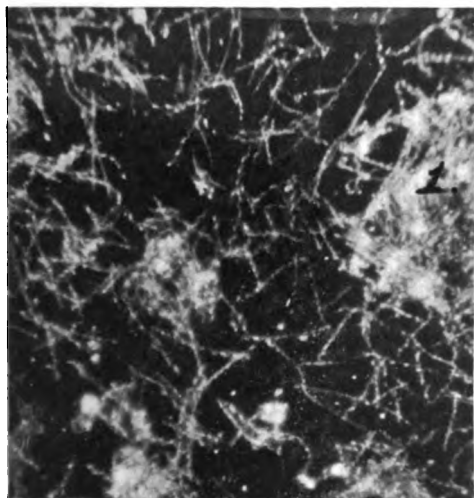
Morphologically *Treponema mucosum* resembles both the pallidum and the microdentium, measuring on the average 8 to 12 microns by 0.25 to 0.3 of a micron (figures 1 and 2, *Treponema mucosum*; figures 3 and 4, *Treponema microdentium*; figures 5 and

6, *Treponema pallidum*). The number of curves varies from six to eight. These curves are remarkably regular and are often quite deep. Both extremities are sharply pointed and often possess at one or both ends a fine, minutely curved projection that varies in length in different organisms. The length of this projection may reach eight to ten microns. The organisms show a graceful rotating movement, and are often seen joined to each other by a thin filament forming a pair, or chain of three, four, or more individuals. Under certain cultural conditions forms suggestive of a longitudinal division are observed.

When cultivated under unfavorable conditions a large number of irregular forms appear. Some of these are almost straight and some are slightly or irregularly curved. There are also many granular particles. These particles may be merely degenerative products or they may be segments which under favorable conditions are capable of reproducing the spirochætæ. These segments or granules take the chromatin stain and vary in size. Not infrequently a long spirochæta is found undergoing a granular segmentation (degeneration?), or a small spirochæta is seen attached to a round body as if it had just sprouted out of the latter. In its staining reactions the mucosum behaves as the microdentium does and takes the red in the Giemsa stain.

Pathogenicity.—When a large quantity of active fluid culture is inoculated into the cutaneous tissue of the skin of a *Macacus rhesus* monkey, it produces an acute inflammation and the tissues remain indurated for one week or ten days. There is no tendency to supuration, and after twenty-four hours the tissue does not contain living spirochætæ. In the testicular tissue of rabbits the inoculated organisms produce also a marked induration (acute inflammatory) which may last for one week, but no abscess formation was observed. The testicle returns to its normal appearance in about two weeks.

When a culture in ascitic agar (as an emulsion) is inoculated, the testicle becomes intensely inflamed within twenty-four hours and remains so for nearly ten days. The inflammation then gradually subsides, but the hard circumscribed nodule persists for many weeks. When the testicle is punctured with a capillary pipette a yellowish thick pus flows out. This pus has a fetid odor and in it a small



number of the spirochætæ are still to be seen. By transplanting the pus into a new culture medium a pure growth of this organism can be obtained. From this fact it seems that the mucosum can survive in the testicle of rabbits when there is simultaneously introduced a foreign substance (agar), but not without the aid of the latter; hence it is not an independent parasite.

Differentiation from the Allied Species.—The mucosum differs from the microdentium in producing a mucin, a stronger fetid odor, a denser growth, and in surviving in the rabbit testicle when introduced with agar. Morphologically it is almost impossible to differentiate these two species. The pallidum differs from this organism by its pathogenicity, by producing no odor or mucin, by its fainter diffuse growth, and by requiring for its growth the presence of fresh tissue in the media.

CONCLUSIONS.

1. A mucin-producing spirochæta has been obtained in pure culture from a case of pyorrhea alveolaris. This organism is an independent species of the genus *Treponema* and, as it is recognized for the first time, I propose for it the name *Treponema mucosum*.

2. Morphologically the mucosum is difficult to separate from the pallidum and microdentium, but, through its biological properties and animal reactions it is easily differentiated from all the rest of the spirochætæ.

3. The mucosum is not parasitic in the strict sense of the term, but exerts a certain pyogenous action when the tissue has been so injured by foreign substances as to enable it to survive.

4. The strong fetid odor in the discharge from pyorrhea alveolaris is due, at least in part, to the presence of the mucosum in the affected tissue.

EXPLANATION OF PLATE 18.

Dark-field views of the organisms. $\times 1,100$.

FIGS. 1 and 2. *Treponema mucosum* from pure cultures ten days old.

FIGS. 3 and 4. *Treponema microdentium* from pure cultures ten days old.

FIGS. 5 and 6. *Treponema pallidum* from pure cultures two weeks old.

THE PURE CULTIVATION OF SPIROCHÆTA DUTTONI,
SPIROCHÆTA KOCHI, SPIROCHÆTA OBER-
MEIERI, AND SPIROCHÆTA NOVYI.*

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATES 19 AND 20.

There are at least four distinct species of spirochætæ that are responsible for the diseases known as relapsing fever. The organisms in the blood of patients suffering from the relapsing fever of Europe were first discovered by Obermeier¹ in 1873, and since then this species has been known as *Spirochæta obermeieri*. The second variety is found in the blood of those who have contracted the disease known as African tick fever; it was discovered in 1904 simultaneously by Ross and Milne,² and by Dutton and Todd.³ This variety bears the name of *Spirochæta duttoni*. In 1905 the third species was discovered in Africa by Koch⁴ who assumed that it was identical with other varieties already known at that time; but the recent investigations of Novy and Fraenkel⁵ indicate that Koch's organism is distinct from the obermeieri or the duttoni. For Koch's organism Novy proposed the name *Spirochæta kochi*. The fourth species, *Spirochæta novyi*, was found by Norris⁶ in 1906 in New York in the blood of a patient with relapsing fever, and was afterwards transmitted to rats and mice. Although very similar in morphology to *Spirochæta obermeieri*, Fraenkel,⁷ Uhlenhuth and

* Received for publication, June 1, 1912.

¹ Obermeier, O., *Berl. klin. Wchnschr.*, 1873, x, 152, 378, 391, 455.

² Ross, P. H., and Milne, A. D., *Brit. Med. Jour.*, 1904, ii, 1453.

³ Dutton, J. E., and Todd, J. L., *Brit. Med. Jour.*, 1905, ii, 1259.

⁴ Koch, R., *Deutsch. med. Wchnschr.*, 1905, xxxi, 1865; *Berl. klin. Wchnschr.*, 1906, xliii, 185.

⁵ Fraenkel, C., *Med. Klin.*, 1907, iii, 928; *München. med. Wchnschr.*, 1907, liv, 201; *Berl. klin. Wchnschr.*, 1907, xlv, 681; *Hyg. Rundschau*, 1907, xvii, 263.

⁶ Norris, C., Pappenheimer, A. M., and Flournoy, T., *Jour. Infect. Dis.*, 1906, iii, 266.

⁷ Fraenkel, C., *Berl. klin. Wchnschr.*, loc. cit.

Haendel,⁸ Schellack,⁹ Strong,¹⁰ Manteufel,¹¹ and others, consider *Spirochæta novyi* to be a distinct species, chiefly on account of the immunity phenomena.¹²

There is another variety which was described by Carter¹³ in India, but it is not yet definitely determined whether or not this is identical with *Spirochæta novyi* or *Spirochæta obermeieri*, although Novy considers it a distinct species on account of its being somewhat thinner than any of the other organisms.

Schellack gives the following comparison of various species:

Spirochæta duttoni: length, 24 to 30 microns; width, 0.45 of a micron.

Spirochæta obermeieri: length, 19 to 20 microns; width, 0.39 of a micron.

Spirochæta novyi: length, 17 to 20 microns; width, 0.31 of a micron.

My success in causing other spirochætæ to grow in culture media made it seem advisable for me to apply similar principles to the blood spirochætæ, *Spirochæta duttoni*, *Spirochæta kochi*, *Spirochæta obermeieri*, and *Spirochæta novyi*, especially as no one had succeeded in obtaining *in vitro* a culture of any of these spirochætæ in which growth was kept up for many generations by transplanting the organisms from culture to culture.¹⁴

There are some investigators who observed a decided increase in the number of the organisms when citrated blood was kept at room temperature or in the thermostat, but they failed to obtain a second generation. According to Novy and Knapp, blood containing the spirochætæ may remain infectious for many days, but no true cultivation is effected.

⁸ Uhlenhuth, P., and Haendel, *Arb. a. d. k. Gsndhtsamte.*, 1907, xxvi, 1.

⁹ Schellack, C., *Arb. a. d. k. Gsndhtsamte.*, 1908, xxvii, 364.

¹⁰ Strong, R. P., *Philippine Jour. Sc.*, 1908, iii, 231.

¹¹ Manteufel, *Arb. a. d. k. Gsndhtsamte.*, 1908, xxvii, 327.

¹² Novy, F. G., and Knapp, R. E., *Jour. Infect. Dis.*, 1906, iii, 291. Breinl, A., and Kinghorn, A., *Lancet*, 1906, i, 668; *Mem. Liverpool School Trop. Med.*, 1906, xx, 61.

¹³ Carter, H. V., *Deutsch. med. Wchnschr.*, 1879, v, 189, 351, 386.

¹⁴ Williams, L. A. and R. S., *Mem. Liverpool School Trop. Med.*, 1906, xxi, 101. Duval, C. M., and Todd, J. L., *Lancet*, 1909, i, 834. Levaditi, C. (*Compt. rend. Acad. d. sc.*, 1906, cxlii, 1099), and Novy, F. G., and Knapp, R. E. (*Jour. Am. Med. Assn.*, 1906, xlvii, 2152), succeeded in keeping up the growth of *Spirochæta kochi* and *Spirochæta novyi* (formerly thought to be the obermeieri by Novy), respectively, for many generations by means of collodion sacs placed in the peritoneal cavity of rabbits; but their work does not enter into consideration in the discussion of cultivation *in vitro*.

As will be described in detail later, I have found the conditions that are necessary for obtaining cultures *in vitro* of the organisms of the relapsing fevers. These conditions are somewhat different from those required by the other spirochætæ I have cultivated. Thus, for the pallidum, microdentium, macrodentium, mucosum, refringens, and phagedenis, a strict anaerobiosis is necessary; while for the relapsing fever spirochætæ the presence of oxygen seems to be essential. My cultivation experiments will be reported in the following pages.

*Material for Cultivation.*¹⁵—Four different strains of spirochætæ were employed for the experiments here described: *Spirochæta duttoni*, *Spirochæta kochi*, *Spirochæta obermeieri*, and *Spirochæta novyi*. All of these have been preserved for many years in rats or mice.

Method of Cultivation.—After trying various culture media and methods I found the following to be the most suitable and reliable for the growth of all of the blood spirochætæ. Into each of a number of sterile test-tubes (I use tubes 2 by 20 centimeters) is placed a piece (not too small) of sterile fresh tissue, usually rabbit kidney, and then are added a few drops of citrated blood from the heart of the infected mouse or rat. Following this, about fifteen cubic centimeters of sterile ascitic or hydrocele fluid are quickly poured into the tubes, and the contents of some of the tubes are covered with a layer of sterile paraffin oil, while the rest are left without the oil. The tubes are now transferred to a thermostat at 37° C.

It is essential to obtain the blood from the infected animal absolutely aseptically. In my experiments, under ether anesthesia, the blood was drawn directly from the heart and was then mixed immediately with citrate solution (1.5 per cent. sodium citrate in physiological saline solution). It is best to obtain the blood between the forty-eighth and the seventy-second hours after the inoculation of the animal. The blood suspension thus prepared was examined for the spirochætæ under the dark-field microscope and was then used for the inoculation of the culture tubes.

¹⁵I am under great obligation to Professor F. G. Novy for placing at my disposal his strains of *Spirochæta duttoni*, *Spirochæta obermeieri*, and *Spirochæta novyi*. The *Spirochæta kochi* employed by me was obtained from Dr. B. T. Terry.

It is very important to employ samples of ascitic fluids which contain no bile, but which form a loose fibrin in the culture tube, for many specimens are unsuitable just because they contain too much bile or do not cause the formation of fibrin when mixed with the fresh tissue in the culture tube. Fluids which have been heated to 56° to 60° C. for thirty minutes, or which have been filtered through the Berkefeld filter are unsuitable. The addition of bouillon or sugar to the fluids also diminishes their cultural value. Sterile tissue kept a day or two on ice is less suitable for cultivation purposes than fresh tissue, and may prove not at all suitable.

It is not necessary to introduce a large number of spirochætæ into the culture tubes. An examination of the culture made immediately after my inoculations usually showed one organism in every twenty or thirty fields, but sometimes it was almost impossible to find a spirochæta even after a long search.

Results of Cultivation.—By the method described above I have succeeded in obtaining pure cultures of the four varieties with which I experimented; i. e., *Spirochæta duttoni*, *Spirochæta kochi*, *Spirochæta obermeieri*, and *Spirochæta novyi*. *Spirochæta kochi* was the first to be cultivated and has already gone through twenty-nine generations within six months. *Spirochæta novyi* was the last to be cultivated and is now in its fourth passage. It may be stated that subcultures can be obtained in the tissue ascitic fluid media by inoculating into it about 0.5 of a cubic centimeter of the original culture. For growth in these subcultures the addition of a small amount of normal rat or human blood is advantageous but not essential.

PURE CULTIVATION OF SPIROCHÆTA DUTTONI.

Rate of Multiplication.—When a small number of organisms is inoculated, the original culture tube shows almost no sign of multiplication within twenty-four hours. On microscopical examination, after forty-eight hours one perceives a slight increase in the number of organisms, but at the seventy-second hour the number of spirochætæ has increased decidedly. Four to six days after inoculation the organisms have increased so much that one may be seen in each field. On the eighth or ninth day the maximum in multipli-

cation is reached, and in every field many actively motile organisms may be seen singly, in chains, or in masses. On the tenth day there comes a sudden change. Not only have the spirochætæ ceased to increase, but a beginning diminution in their number may be observed. The active organisms are very few and the other spirochætæ seem to be on the verge of disintegration. The spirochætæ are seen to lose their protoplasm, and the spiral framework of the body is left bare. Many spherical bodies and irregular protoplasmic masses make their appearance. In cultures which are not covered with oil and which are more than fifteen days old it is exceptional to find an active spirochætæ, but I should mention that the spirochætæ multiply somewhat more slowly and disappear also somewhat later in the tubes in which the contents are covered with paraffin oil than in the tubes containing no oil.

At 15° C. no multiplication takes place and the organisms undergo degeneration very promptly. No growth is obtainable in an atmosphere of hydrogen or *in vacuo*.

Effect upon the Culture Media.—Apparently there is no visible alteration in the culture media in which the spirochætæ are rapidly multiplying. The tissue may look somewhat paler, but no coagulation of the fluid (ascitic or hydrocele) takes place, and no odor is produced by the growth.

Transplantations.—Subcultures can be made at any time between the fourth and ninth days. It is not advisable to make the subinoculations earlier than the fourth day or after the culture has passed its period of maximum growth. By using about 0.5 to 1 cubic centimeter of the culture any number of successive transfers (so called passages) may be obtained. It is extremely important that no bacterial contamination should occur, because the simultaneous growth of certain cocci or bacilli causes the spirochætæ to disappear from the culture.

Morphology.—(Figures 1 to 5.) In young cultures short forms with only two or three curves are very numerous. In the cultures approaching the maximum growth (eighth to ninth day) the length of the organisms is about that seen in normal infections. The curves are very regular and deep. The spirochætæ may be single, paired, or in chains. They are vigorously motile. They usually show a

long, regularly curved, delicate projection at each end of the body. In old cultures, after ten days, the spirochætæ lose their motility and the protoplasmic masses concentrate at varying points along the spiral skeleton. There are many organisms which show one or more spore-like spherical bodies attached laterally at irregular intervals (figure 2). In such old cultures there are usually many spiral skeletons entangled or embedded in granular protoplasmic remains (figure 5). But even in these old cultures a few active spirochætæ are occasionally met with. Old cultures may be temporarily rejuvenated, *i. e.*, for three or four days, by introducing into them fresh ascitic fluid and a piece of sterile fresh tissue, but degeneration that is almost complete soon follows.

It is interesting to notice that the spirochætæ grown under a layer of paraffin oil are somewhat thinner than those grown in the absence of oil.

Mode of Division.—(Figure 4.) In luxuriantly growing cultures it is difficult to decide whether the spirochætæ undergo longitudinal or transverse division, as they are usually seen in pairs or chains, united to each other by a thin filament. Their general appearance suggests transverse fission, but no undoubted instance of transverse division has thus far been observed. At the same time there are always a few instances of unmistakable longitudinal division. Moreover, I have repeatedly found cultures in which, probably due to certain unfavorable conditions, almost every organism showed partial longitudinal division. When these organisms were watched for some time under the dark-field microscope, the process of longitudinal division could be observed.

Pathogenicity.—*Spirochæta duttoni* cultivated *in vitro* remains infectious for rats and mice. I have been able to infect rats and mice with cultures that were in their ninth passage, and as far as the severity of the infection is concerned no difference has been seen between that caused by cultures and that caused by inoculating blood obtained from infected animals. But, as will be pointed out when *Spirochæta kochi* is discussed, it is possible that a diminution in the virulence may yet be observed when the number of passages has become sufficiently large.

PURE CULTIVATION OF SPIROCHÆTA KOCHI.

Spirochæta kochi is the first variety of the spirochætæ of relapsing fever that was successfully cultivated by me. In most respects it is very similar to *Spirochæta duttoni*. *Spirochæta kochi* has been growing *in vitro* since February, 1912, and by renewing the cultures regularly every week no difficulty has thus far been experienced in keeping the culture growing. As a rule, the maximum growth is reached on the ninth day. As in the case of *Spirochæta duttoni*, no visible alteration of the media results from the growth and there is no perceptible odor at any period of its cultivation. Morphologically (figures 6 to 11) it is almost indistinguishable from *Spirochæta duttoni* except that it is possibly a trifle thinner than the latter. It divides longitudinally and possibly also transversely.

Pathogenicity.—*Spirochæta kochi* cultivated *in vitro* retained for several passages all its usual virulence and killed rats and mice fairly constantly, but when tested after the tenth passage *in vitro* it no longer killed these animals. In the rats and mice that had been inoculated, spirochætæ developed in large numbers, but their appearance was delayed to the third or fourth day. The sixteenth passage also caused an infection in these animals, but was apparently less virulent than the tenth passage. From this it appears that the virulence of these organisms is being gradually attenuated by prolonged cultivation *in vitro*. The fourth passage of the culture failed to infect rabbits, guinea pigs, cats, dogs, a chicken, or a *Macacus rhesus* monkey.

PURE CULTIVATION OF SPIROCHÆTA OBERMEIERI.¹⁶

Spirochæta obermeieri multiplies more rapidly in cultures *in vitro* than *Spirochæta duttoni* or *Spirochæta kochi*. Within twenty-four hours there is a decided increase in the number of organisms in the culture and the maximum growth is usually reached on the seventh day, when every field shows several spirochætæ. The decline commences after the seventh day, and within the next two days degeneration sets in. Under the cover of paraffin oil the organisms grow more slowly and remain active for a day or two longer than in the tubes containing no oil.

¹⁶ Synonymous with *Spirochæta recurrentis*.

Transplantations.—Successful subcultures are best obtained by inoculating new media with about one cubic centimeter of a culture that has shown active growth for three to four days, although a culture at the height of its growth (seventh day) can also be used.¹⁷ I have found it always advantageous to provide two series of tubes, —one with and the other without the paraffin oil layer, because not infrequently the growth is more abundant and vigorous in one than in the other of these two series. I have been unable to determine the cause of this irregularity, but there is no difficulty in continuing the cultivation if both conditions are provided. No growth takes place at room temperature.

Morphology.—(Figures 12 to 17.) In young cultures (not older than three or four days) there are, besides the active specimens of average length and width, numerous very motile short organisms which show only two or three curves. Among these forms one frequently encounters the skeletons of degenerated organisms, which were no doubt introduced into the culture with the inoculation material and died afterwards. In regard to their general appearance there is a striking contrast between the spirochætæ found in the blood of an infected mouse or rat and those seen in young cultures. The former are quite regular in their curves and length. The cultivated organisms are usually irregularly wavy and shorter. The movements are similar in both; at one time there is a serpentine forward movement and at other times there are violent lateral vibrations which involve the middle portion of the body and both ends. In older cultures (six or seven days) the lengths of the organisms are more uniform and approach the standard observed in specimens from the blood, but the curves are still shallower and more irregular. The organisms may be entangled in large masses or may be found in pairs or in chains (figure 14). Some specimens have blunt ends to which a mass of minute refractive granules are attached (figure 16). In this stage every organism is actively motile.

The cultures which have just passed their maximum growth enter a period of decline or degeneration. These cultures contain num-

¹⁷ The addition of several drops of fresh, undefibrinated, normal rat blood favors the growth decidedly.

erous motionless forms which have irregular concentrations of protoplasm at various portions of the spiral skeleton. To many organisms are now attached one or more spherical bodies measuring about 0.7 of a micron in diameter and resembling spores. These bodies are, however, not within the protoplasm, but are attached to the sides of the spirochætæ. Free spherical bodies may also be found. There are many skeletons of spirals which show no longer any masses of protoplasm. Sometimes these degenerated forms are closely bound together by a mass of granular protoplasm that varies in size and form. Occasionally quite normal active spirochætæ are seen.

If left alone the degenerative changes just described proceed rapidly and within three or four days almost no spirochætæ with protoplasm can be found. On the other hand, if fresh tissue and ascitic fluid are added, one can rejuvenate these cultures for two to three days, but under these conditions the vigor of the multiplication is limited and feeble.

Mode of Division.—(Figure 15.) In pure cultures *Spirochæta obermeieri* multiplies by longitudinal division, and possibly also by transverse fission. The longitudinal division occurs in almost every organism whether it be single, paired, or in a chain, and irrespective of the length of the spirochæta. As stated already, the spirochætæ cultivated *in vitro* are less curved than those found in the blood. They are usually somewhat broader, especially when showing longitudinal division. The organisms which are about to divide are somewhat less vigorously motile. The first sign of division is the appearance at any part of the organism of a short narrow cleft parallel to the axis of the spirochæta. Within a few seconds another short cleft comes into view at some distance from the first one; then, after short intervals, a third and fourth cleft are seen. The organism now appears as if its two halves were connected with each other by several protoplasmic bridges of varying width. At the same time each of the halves begins to jerk in a peculiar way, the movements of the halves being alternately in opposite directions. The positions of the several clefts change, one taking the place of another. The fluid character of the protoplasmic bridges which connect the two halves is thus suggested. Finally two clefts fuse

together into a longer cleft, and into this a third and fourth cleft are gradually merged. The organism is now split into two parts, except for the extremities, which in time divide completely. Thus two spirochætæ are formed from one, and, as a rule, one of them dashes away immediately. In some instances the newly formed daughter cells are held together by a thin filament. On the other hand, a transverse division seems also to occur. This mode of division may be indicated by the presence of two or more organisms joined together by a thin filament. It frequently happens that the length of one member of the pair of spirochætæ thus connected is much greater than the other. This appearance suggests that the pair was formed by the transverse division of a longer individual. Nevertheless, the frequency of the occurrence of the transverse fission in culture must be less than that of longitudinal division, because the latter can be seen in any multiplying culture, while an absolutely convincing case of the former has not been observed by me.

Pathogenicity.—The culture of *Spirochæta obermeieri* retains its virulence for rats and mice as was proved by the results of inoculating these animals with the seventh passage *in vitro*. Whether or not the virulence may gradually become attenuated after the cultivation has been continued for a long time will have to be determined later.

PURE CULTIVATION OF SPIROCHÆTA NOVYI.

This variety is now recognized as a distinct species, chiefly through the studies of Fraenkel, Schellack, Uhlenhuth and Haendel, and others. Their conclusions are based upon immunity phenomena, although certain morphological peculiarities are also pointed out. It was first noticed by Schellack that *Spirochæta novyi* resembles *Spirochæta obermeieri* more closely than it does the African varieties, but it is somewhat thinner than the *Spirochæta obermeieri*.

Norris, Pappenheimer, and Flournoy, as well as Novy and Knapp,¹⁸ observed a certain multiplication of this organism in citrated blood kept at body temperature, but a second transfer

¹⁸ In their early articles this variety was dealt with as *Spirochæta obermeieri*, as there was still no differentiation established between these two species. Schellack in 1908 was the first to give this organism the name of *Spirochæta novyi* and separate it from the obermeieri.

showed no further growth. Briefly stated, no culture in the strict sense of the term was obtained.

In my experiment I obtained a growth of this organism in the same kind of medium as that in which the other three strains were cultivated. I have, however, experienced more difficulties with this variety than with the rest, but have at last succeeded in getting it to grow *in vitro*. It is now in its fourth passage.¹⁹

The general characteristics (figures 18 to 21) of the culture are almost identical with those of *Spirochæta obermeieri* except that the novyi is somewhat thinner than the latter. It shows longitudinal division as well as forms suggestive of transverse division. Up to the present the virulence of the organism growing *in vitro* is comparable to that of the spirochætæ contained in the blood of infected rats and mice.

SUMMARY.

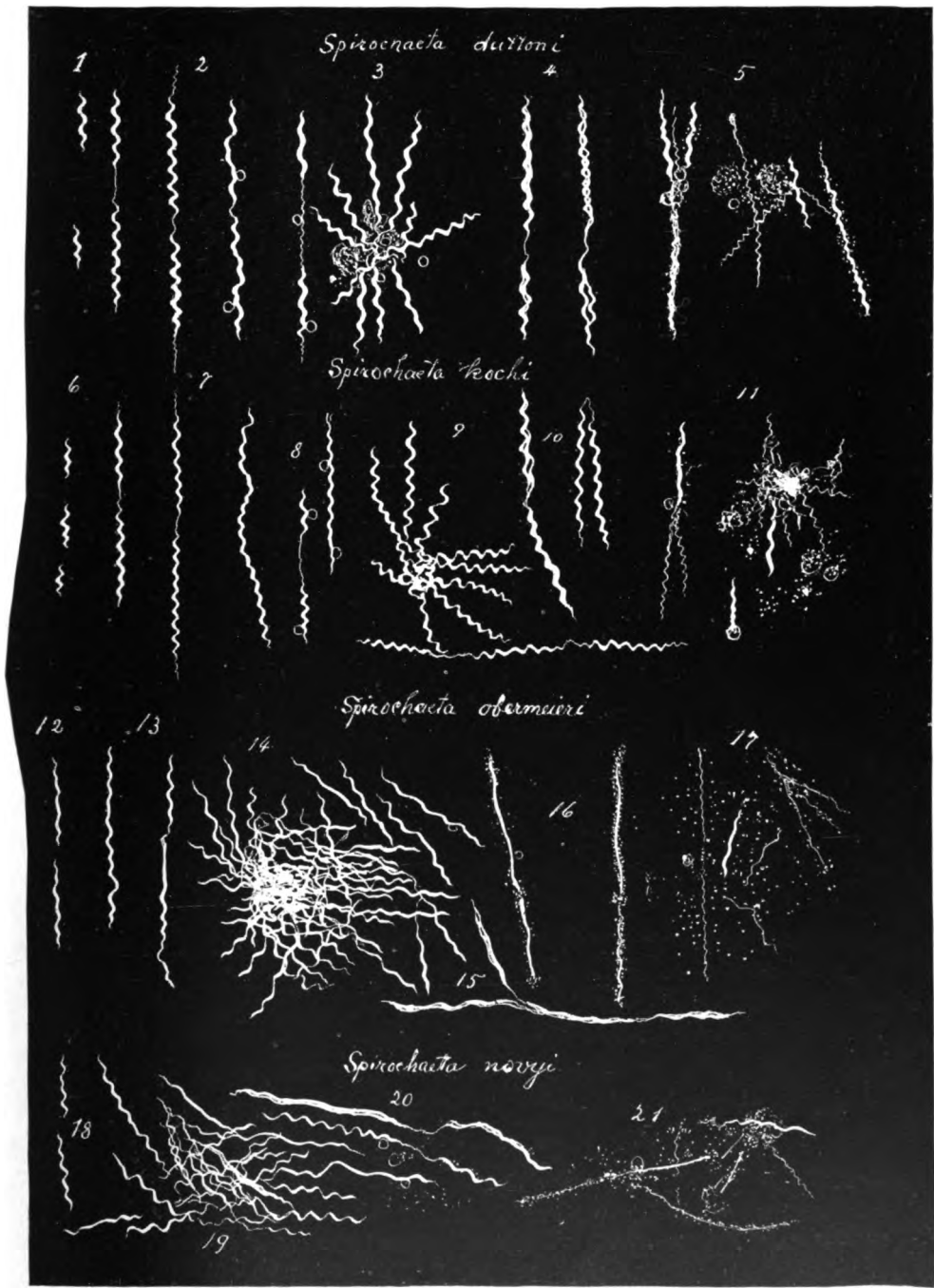
1. A method for the pure cultivation of *Spirochæta duttoni*, *Spirochæta kochi*, *Spirochæta obermeieri*, and *Spirochæta novyi* is described in this paper. *In vitro* these strains reach their maximum growth after seven, eight, or nine days at 37° C. For their multiplication they require the presence of a piece of fresh sterile tissue and a body fluid capable of forming a loose fibrin with the tissue. The presence of some oxygen seems indispensable for their growth, since they fail to grow in an atmosphere of hydrogen or *in vacuo*. No growth was obtained at room temperature.

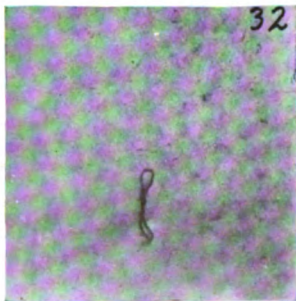
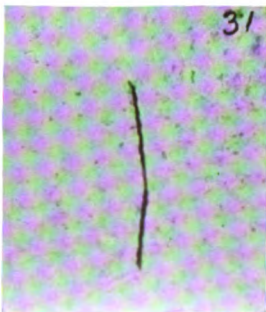
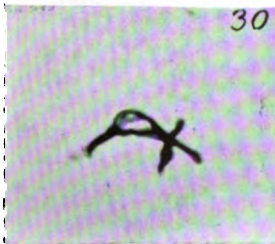
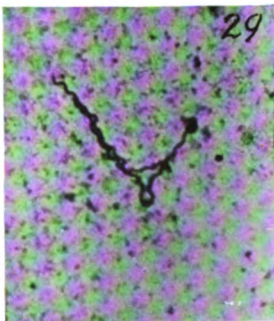
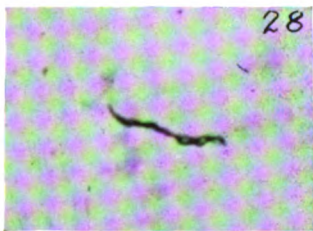
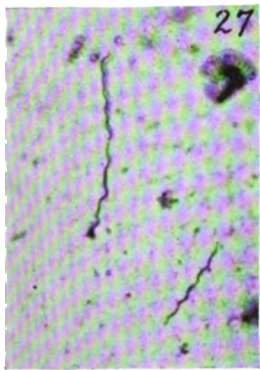
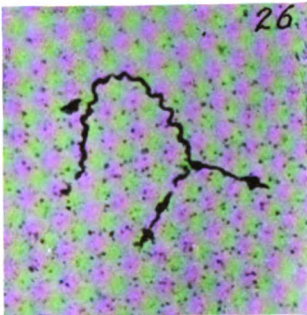
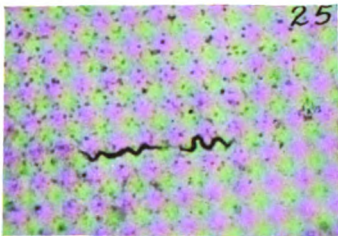
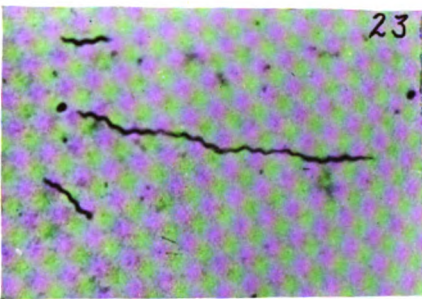
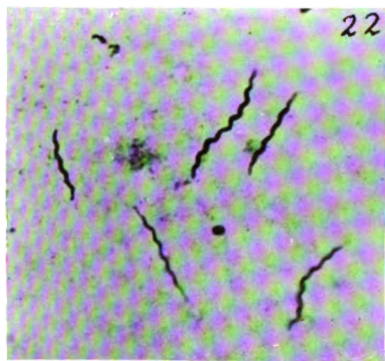
2. From cultures that show a good growth subcultures can be made and the growth can be kept up in this way for many passages.

3. The pathogenicity of these organisms is not lost by cultivation, although there is a tendency for the virulence to become attenuated after growth *in vitro* has continued for a long time.

4. Longitudinal division has been observed and was followed under the dark-field microscope in fresh preparations from cultures. It occurs in all the species irrespective of the length of the organism. Transverse division seems also to occur, but I have not yet observed the entire process.

¹⁹ A more vigorous growth in subcultures can be obtained by adding several drops of fresh, undefibrinated, normal rat blood to the media.





EXPLANATION OF PLATES.

PLATE 19.

Series A.—Schematic reproductions of the spirochætæ under the dark-field microscope.

FIGS. 1 to 5. Different stages of growth of *Spirochæta duttoni* in pure culture. Fig. 1, young forms; Fig. 2, average forms with spore-like bodies in one of the spirochætæ; Fig. 3, a mass of spirochætæ; Fig. 4, longitudinal division; Fig. 5, degeneration forms.

FIGS. 6 to 11. Different stages of growth of *Spirochæta kochi* in pure culture. Fig. 6, young forms; Fig. 7, average forms; Fig. 8, spirochætæ with spore-like bodies; Fig. 9, an entangled mass of the spirochætæ; Fig. 10, longitudinal and transverse division; Fig. 11, degeneration phase.

FIGS. 12 to 17. *Spirochæta obermeieri* in pure culture. Fig. 12, young forms; Fig. 13, usual forms; Fig. 14, a mass of growing spirochætæ; Fig. 15, longitudinal and transverse division; Fig. 16, blunt forms with granulation; Fig. 17, degeneration phase.

FIGS. 18 to 21. *Spirochæta novyi* in pure culture. Fig. 18, young forms; Fig. 19, average forms; Fig. 20, longitudinal and transverse division; also a few spore-like bodies; Fig. 21, degeneration stage.

PLATE 20.

Series B.—Microphotographs of the spirochætæ from the preparations fixed in sublimate alcohol and stained for twelve hours with the Giemsa solution. $\times 1,100$.

FIGS. 22, 23, and 24. *Spirochæta duttoni* from pure cultures (fourth generation). Fig. 22, average forms; Fig. 23, one long and two short forms; Fig. 24, an entangled mass.

FIGS. 25 and 26. *Spirochæta kochi* from pure cultures (twenty-fourth generation). Fig. 25, young forms; Fig. 26, near the time of decline (tenth day).

FIGS. 27, 28, 29, and 30. *Spirochæta obermeieri* from pure cultures (fourth generation). Fig. 27, an average and a small form; Fig. 28, a doubled-up form; Fig. 29, entangled spirochætæ near the time of decline (eighth day); Fig. 30, a peculiar form consisting of partially fused spirochætæ, probably a result of imperfect longitudinal division in succession. This often occurs in a less favorable culture medium. It is not an artifact produced during the fixation, because it can be seen in a fresh preparation under the dark-field microscope; each individual of the mass is actively motile.

FIGS. 31 and 32. *Spirochæta novyi* from pure cultures (fourth generation). Fig. 31 shows the average forms, and Fig. 32 shows a doubled-up specimen.

A METHOD FOR CULTIVATING TREPONEMA PALLIDUM IN FLUID MEDIA.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Treponema pallidum is an obligatory anaerobe which together with certain other spirochætæ forms a special class of organisms whose growth requires the presence of a fresh, sterile tissue in culture media.¹ The growth of *Treponema pallidum* in any suitable medium is very slow, and continues for a considerable length of time. In a solid medium, for which the pallidum shows a particular preference, the growth continues for about two months after transplantation. On the other hand, the cultivation of the pallidum in a fluid medium, with the addition of fresh tissue, by means of an anaerobic jar in which oxygen is removed through the combination of vacuum, displacement with hydrogen and absorption by pyrogallol, has given rather inconstant results. In one series of cultivations the growth may be quite favorable, while in others it may fail altogether. The loss of time and material from unsuccessful fluid cultivations is very great and the entire procedure is therefore in need of improvement.

The method which I am about to describe in detail has already proved to be entirely satisfactory and possesses certain special features that are lacking in the usual anaerobic cultural methods now in general use. In the case of the usual method, an ordinary test-tube is filled with the fluid in which the cultivation of an organism is intended, the fluid is then inoculated, and cultivated in a sealed jar under anaerobic conditions. The process is quite satisfactory for the cultivation of most anaerobic bacteria, but, as has already been mentioned, it is unreliable for obtaining growth of the

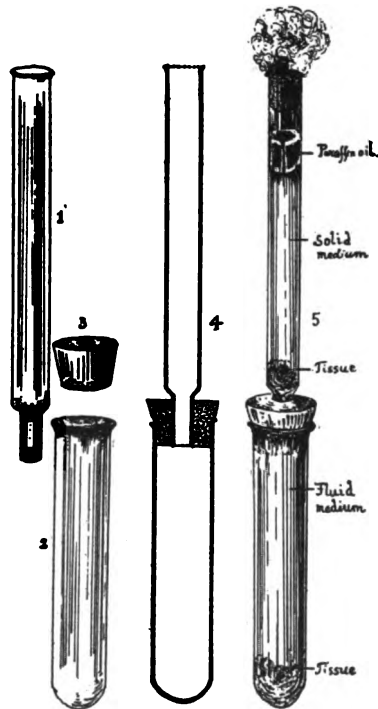
¹ It may be stated that the addition of sterile fresh tissue to a fluid medium alone is not only inadequate to make the pallidum grow, but is unable to keep it alive for any length of time. In this respect the pallidum is very different from the obligatory anaerobic bacteria, as the latter grow abundantly in such a medium.

pallidum in a fluid medium even with the addition of fresh tissue. The failure to effect cultivation of the pallidum by this method is doubtless due to the fact that with this process the removal of oxygen is often too incomplete to enable the inoculated pallidum to grow or even to survive the time required by the fresh tissue to absorb the remaining oxygen and render the fluid perfectly anaerobic. In other words, *Treponema pallidum* is too delicate an organism to survive the unfavorable conditions which last for some time within the fluid in which it is freshly transplanted. The source of this difficulty can, however, be removed by providing the organism with a more suitable medium in which it can at once begin to adjust itself. A solid medium consisting of a mixture of ascitic fluid and agar with the addition of a fresh, sterile tissue fulfills this requirement. Thus the new method which is designed to exclude this difficulty is composed of two distinct parts; one for the solid and the other for the fluid cultivation. By combining both the solid and the fluid culture media for simultaneous cultivation, the shortcoming of the latter is easily supplemented by the former which is highly suitable for the growth of the pallidum. By this method the pallidum grows at first in the solid medium as usual, and then, when the cultural conditions in the fluid portion become favorable, it migrates from the solid portion into the fluid and multiplies there abundantly.

A solid medium in this method not only serves as a reservoir of the growing spirochætæ for the fluid culture, but it is also a good indicator for determining the purity of the culture; for should any contamination occur, it is infallibly indicated by the development of the colonies of bacteria in the agar column, while it is quite difficult to detect a contamination with certain bacteria in a fluid medium alone. As will be seen later, the entire process of cultivation is carried on without the aid of a sealed jar, vacuum, or pyrogallol. The state of growth, or any changes that may take place in the medium, can easily be observed at any time without disturbing the cultivation, and, if desired, the content of the culture may, under aseptic precautions, be drawn out and examined. These are the features which may be regarded as special advantages of the new process in comparison with the older procedures.

METHOD.

Construction of the Culture Tube.—This consists of a test-tube, 1.7 centimeters wide and 20 centimeters long, whose bottom ends in a hollow projection made by fusing a short piece of a strong glass tubing, 0.7 of a centimeter bore, to the perforated bottom (text-figure, 1); a large test tube, 2.5 centimeters wide and 15 centimeters long (text-figure, 2); and a perforated rubber cork, size 5 (text-figure, 3). These parts are combined by connecting the two tubes by means of the rubber cork (text-figure, 4). The



TEXT-FIG. For description see text, p. 36.

upper tube is intended for the solid, and the lower for the fluid culture medium.

Preparations for Cultivation.—The double tube just described is thoroughly cleaned and dried; the upper tube is then plugged with non-absorbent cotton. Just before use the whole is sterilized in an

autoclave. In the meantime pieces of sterile, fresh rabbit kidney are prepared. A slightly alkaline agar (2 per cent.) is also freshly prepared, autoclaved, and kept in a fluid state. A sufficient quantity of sterile paraffin oil should be kept on hand.

Process of Cultivation.—1. After the double tube cools, one or two pieces of the sterile, fresh tissue are put into the lower tube. This is done by removing the rubber cork and the upper tube together, which are quickly replaced and fitted as tightly as possible. These parts must not be loosened again. Another piece of tissue is now put into the upper tube. The tissue goes down to the perforated bottom of the tube, but it should be large enough not to pass through the connecting hole into the lower tube.

2. After tissues have been placed in both tubes, the lower tube is filled with ascitic fluid or a mixture of ascitic fluid and bouillon. This is done by means of a large, sterile bulb pipette whose tubular portion is so drawn out as to pass through the connecting tube with a margin of space that permits the air that is displaced by the fluid to escape. The lower tube must be filled up until there is no air bubble inside the tube.

3. The inoculation of the pallidum is next made. By means of a long capillary pipette, made by drawing a sterile glass tubing, a sufficient quantity of a well growing culture is aspirated and used for inoculation of the double tube. For this purpose a syringe with a connecting pressure rubber tubing is recommended. First the capillary pipette is inserted into the fluid of the lower tube into which some of the contents are forced; then the remaining portion of the culture in the pipette is emptied into the upper tube just about the tissue.

4. When the inoculation has been performed, the upper tube is filled with a solid medium. The medium is prepared by mixing one part of ascitic fluid with two parts of slightly alkaline agar in a sterile flask. The mixing is done when the temperature of the melted agar is about 42° C. Before solidification of the ascitic agar sets in, the medium is quickly distributed into the upper tube. The quantity of the medium is about fifteen cubic centimeters.

5. The last step is to add an arbitrary amount of sterile paraffin

oil in order to cover the surface of the solid medium. About three cubic centimeters suffice (text-figure, 5).

6. The culture is then incubated at 37° C.

For the sake of clearness I have described the procedure as if I were dealing with a single set of double tubes, but in practice it is my custom to employ twelve sets at a time. The advantages of using several sets at a time are twofold. The first is an economic advantage: when one kidney is removed from a normal rabbit it usually furnishes enough tissue to fill at least six sets. As I usually sacrifice one rabbit, I have enough tissue for a dozen sets. The second advantage in making several sets is that, owing to the numerous steps that must be taken in making one series of cultivation, the possibilities of contamination are very great and it is best to provide against this accident so as to insure success.

As regards the capacity of the culture tubes, it is understood that it depends altogether upon the purpose of the work and whether or not a large quantity of the culture is desired. As the nature of my work requires as large a quantity of the material as I can obtain, I use comparatively large tubes.² But, according to my experience, it is not advisable to employ still larger tubes, as the result may be an enormous waste of material when accidental contamination takes place.

The method described in this article is especially adapted for the pure cultivation of *Treponema pallidum*, *Treponema microdentium*, *Treponema macrodentium*, *Treponema refringens*, *Treponema mucosum*, and *Treponema pertenue* in fluid media, when these organisms have been growing already in solid media. It is unsuitable for obtaining the growth of these spirochætæ when they are associated with other bacteria.

SUMMARY.

1. A method is described for obtaining pure cultures of *Treponema pallidum* and allied species of spirochætæ in fluid media.
2. The principle of the method is based upon the superposition of a favorable culture medium upon a less favorable one.
3. The method here described is not suited to the growth of spirochætæ when they are admixed with contaminating bacteria.

² The fluid culture of *Treponema pallidum* is used for the preparation of luetin as well as for immunity studies.

THE ACTION OF SUBDURAL INJECTIONS OF EPINEPHRIN IN EXPERIMENTAL POLIOMYELITIS.

By PAUL F. CLARK, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

In 1903 Meltzer¹ showed that a subcutaneous injection of epinephrin caused a marked change in a local inflammation such as is produced by inoculating cultures of *Staphylococcus aureus* or a drop of turpentine into the soft tissues of the rabbit's ear. The effect consists in a contraction of the actively hyperemic vessels at the periphery of the inflammatory area, while the more severely injured vessels within the inflamed focus remain unaffected. The contraction diminishes the transudation of fluid, the so-called lymph, from the hyperemic vessels, and thus reduces the local edema.

The lesions of poliomyelitis are associated with profound alterations of the blood-vessels, and are attended by transudation of fluid and emigration of white corpuscles from the altered vessels. The degree of vascular and interstitial changes varies in intensity in different cases and at different levels of the spinal cord. The vessels immediately within the focus of main injury are severely, while those at the margin of injury are often slightly affected. It is characteristic of lesions of certain cases of poliomyelitis in man and also in the monkey, although to a less extent, to be progressive, while in still other cases the lesions become quickly localized. This progressive tendency is most serious and alarming when the paralysis is of the ascending type and threatens the origin of the nerves controlling respiration and in especial those that supply the diaphragm. There is reason to believe that in the course of the ascent of the lesions slighter hyperemic states of the vessels attended with exudation precede the severer alterations of the vessels in which the

1. Meltzer, S. J., and Meltzer, Clara: Jour. Med. Research, 1903-1904, x, 135.

perivascular cellular infiltration is extreme and the hemorrhagic eruptions large.

In view of the pathology of poliomyelitis as briefly outlined, the hyperemic vessels at the periphery of the lesions should be subject to the influence of the contracting effects of epinephrin. This response of the vessels should bring about a cessation of the exudation through which the dangers arising from the presence of an inflammatory edema on adjacent nerve cells, which themselves are not the seat of direct injury, may be averted. Such an action might come to be very important and even life-saving in cases in which the nerve-cells that preside over the function of the phrenic nerves are involved. This temporary benefit could conceivably be converted into a permanent one by the cessation of the ascending lesion through the ordinary processes of limitation that are constantly encountered in cases of human poliomyelitis.

Moreover, the peculiar action of epinephrin on the vessels in the actively hyperemic area in contradistinction to the center of inflammation will suffice to indicate the nature of the pathologic process responsible for the ascent of the lesions. If the ascent arises from a continuous involvement of nerve-cells, epinephrin will be without any effect; but if it is produced by consecutive vascular involvement, then it may bring about a definite ameliorative action.

That epinephrin can be injected into the subdural space without danger to life has been shown through its employment in combination with cocain in the production of spinal anesthesia. Auer and Meltzer² have recently shown that the subdural injection of suitable quantities of epinephrin in the monkey does no harm and produces a characteristic action on the blood-pressure. This action consists in a slow but considerable rise and a gradual fall of the pressure. The duration of the rise is longer than after an intravenous injection, in some instances more than half an hour. The fall of blood-pressure occurs so slowly at times that the original level is not reached during a period considerably over an hour. Incidentally it may be mentioned that Meltzer³ has found that epinephrin is destroyed by the cerebrospinal fluid taken from patients with poliomyelitis.

2. Auer, J., and Meltzer, S. J.: Proc. Soc. Exper. Biol. and Med., 1912, ix, 79.

3. Meltzer, S. J.: Proc. Soc. Exper. Biol. and Med., 1911, ix, 27.

At Dr. Meltzer's suggestion I have studied the action of subdural injections of epinephrin⁴ on monkeys paralyzed after intracerebral inoculations of the virus of poliomyelitis. The virus employed is one that invariably causes a fatal ascending paralysis or a rapidly fatal paralysis of respiratory centers in the medulla. In the former instance death results from the inclusion in the ascending process of the nerve-cells from which the phrenic nerves arise. The animals selected were such as were already extensively paralyzed or were moribund and would have survived only a short time longer. They were limp, lay without movement, except for superficial respiratory movements of the chest, and they were usually in a semi-conscious or even unconscious state. The life of none of these monkeys was actually saved, but it was often greatly prolonged,⁵ while the effects of the epinephrin on the general condition of the dying animals were often remarkable. I shall now give in brief form a few illustrative protocols.

Protocol A.—Macacus rhesus. Nov. 9, 1911: Animal completely prostrate, moribund; breathing is feeble, slow, almost entirely diaphragmatic; only slight response to mechanical stimulation; eye reflexes slight. At 1.30 p. m.: 1.5 c.c. of 1:1,000 solution epinephrin injected subdurally. At 3 p. m.: Breathing more rapid and deep; good response to mechanical stimulation; eye reflexes stronger. The animal appears bright. At 4 p. m.: In response to mechanical stimulation the left arm is moved inward and to some extent in the upward direction. The animal is bright and entirely conscious. The improvement in the paralysis can be followed from above downward. Animal turned on left side, the less paralyzed side. At 5 p. m.: No heart beat; pupils dilated; animal dying from asphyxia.

Protocol B.—Macacus rhesus. Feb. 19, 1912: Back, right arm, and leg paralyzed; prostrate; tremor of head; excitable.

February 20: Animal moribund; breathing feeble and shallow; little tonus in arms or legs; semi-conscious. At 10 a. m.: 1.5 c.c. of 1:1,000 epinephrin injected subdurally. No immediate effect. At 10.30 a. m.: Consciousness has returned and respirations are accelerated. The animal eats part of a banana offered. At 10.45 a. m.: Respirations deeper. At 11 a. m.: Marked increase in the tonus of arms and legs; some voluntary motion. At 12 m.: Animal appears bright, raises the head, and uses all of the limbs somewhat; respirations rapid

4. The brand of epinephrin used in the studies was the adrenalin chlorid of Parke, Davis & Co.

5. See Flexner and Lewis, (Jour. Exper. Med., 1910, xii, 227) for the clinical history of cases of experimental poliomyelitis in the monkey.

but fairly deep. At 1 p. m.: Voluntary movements greater. At 3 p. m.: Marked improvement; respiration about normal. The animal takes food eagerly. At 4 p. m.: The tonus in the arms is diminishing; animal still bright. At 4.30 p. m.: 4 c.c. of 1:2,000 epinephrin injected.

February 21: At 9 a. m.: The animal is still bright and eats, but the muscles of the legs have lost in tonus. Uses right arm somewhat and left to a less degree. The condition persisted unchanged throughout the day.

February 22: Animal remains bright; has eaten a whole banana. The extremities still show tonus.

February 23: Animal is growing weaker and appears less bright. At 12 m.: 1 c.c. of 1:1,000 epinephrin injected. No marked improvement.

February 24: Monkey developed a severe diarrhea during the night. Weaker and less tonus in arms and neck. At 12.30 p. m.: 2 c.c. of 1:2,000 epinephrin injected subdurally. At 7 p. m.: Animal weaker. Diarrhea continues.

February 25: The animal gradually failed and died in the morning.

Protocol C.—Macacus rhesus. Feb. 24, 1912: Animal prostrate; little tonus in either legs or arms. The left leg and both arms respond slightly to mechanical stimulation; paralysis of the muscles of the neck; respirations feeble. At 1 p. m.: 1.5 c.c. of 1:1,000 epinephrin injected subdurally. At the end of half an hour there is no noticeable change. Improvement sets in a little later, and at 7 p. m., the animal appears bright, the neck muscles stronger, and the respiration improved.

February 25: The paralysis of the limbs has increased; the respirations have become somewhat more shallow but are better than before the injection. A second injection of 1 c.c. of epinephrin was given, but without producing pronounced change.

February 26: The animal was found dead in the morning.

Protocol D.—Macacus rhesus. March 4, 1912: Arms and back weak, but not completely paralyzed; the left arm and leg weaker than the right. At 10 a. m.: 1 c.c. of epinephrin injected subdurally. At 6 p. m.: No evident improvement following the injection; the paralysis has progressed somewhat.

March 5: The animal is prostrate; back, neck, and four limbs all paralyzed, but not completely. At 10.15 a. m.: Epinephrin 1.5 c.c. injected. At 12 m.: The animal is brighter, eats; the muscles show increased tonus; respiration strong.

March 6: This morning the respiration is somewhat irregular, fifty-four to the minute; monkey cannot move arms or legs. The respiration is still fairly strong and both the intercostal muscles and the diaphragm are in use. At 11.30 a. m.: 1.5 c.c. of epinephrin injected, and at 5 p. m.: 1 c.c. of epinephrin injected, without producing any change in the extent of the paralysis of the extremities.

March 7: The respiration has become more feeble. At 12 m.: 1.5 c.c. of epinephrin injected. At 4 p. m.: The respiration is almost wholly diaphragmatic and is feeble and shallow. 1.5 c.c. of 1:1,000 epinephrin injected subdurally.

At 5 p. m.: Respiration shallow, expiration forced.

March 8: During the night the animal has improved; the respiration is not forced. At 11.30 a. m.: 1.5 c.c. of epinephrin injected; no obvious response to this injection. The animal is gradually becoming more feeble.

March 9: Respirations shallow and labored; expiration forced. At 10.30 a. m.: 1.5 c.c. of epinephrin injected. At 10.45 a. m.: Respirations become deeper and more rapid, but forced expiration still continues. At 5 p. m.: The animal died.

The illustrative protocols given indicate that a subdural injection of epinephrin is capable of producing a marked change in the character of the paralytic phenomena in experimental poliomyelitis, although the effects are not equally striking in all cases. The general result was an improvement in the muscular tonus of the paralyzed muscles and in the respiratory movements. In some animals (Protocols A, B and D) the effects were striking, and a state of extreme flaccidity and unconsciousness, with almost complete disappearance of reflexes, was succeeded by one of tonus, of strengthened reflexes and of return of consciousness. Life was undoubtedly prolonged in these cases. In the other case (Protocol C) there is no definite proof that life was prolonged, and the symptoms were only moderately ameliorated. An examination of the two classes of cases would seem to show that the employment of the epinephrin relatively early in the course of paralysis does not in the monkey inoculated intracerebrally with a highly active virus bring about an arrest of the progress of the disease. The life-saving action of the epinephrin is shown in the case of the moribund animals, in which life was prolonged either for several hours or for several days by the restoration of the failing respiratory function.

Finally, the effects of epinephrin in the experimental poliomyelitis support the view that a state of hyperemia of the blood-vessels, attended by an exudation of plasma and probably of cells also, precedes the severer state of destruction of nerve-cells and interstitial tissue of the spinal cord. They indicate further that subdural injections of epinephrin in proper doses may be found capable of averting in human beings, the subjects of ascending forms of poliomyelitis, a fatal issue through the involvement, in the extending hyperemia and inflammatory edema, of the nerve-cells from which the phrenic nerves take their origin. Should this temporary interruption of the active pathologic process coincide with the natural limitation of the disease, even life may be spared. The experiments do not indicate that epinephrin itself contributes in any way

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to the promotion of the limitation of the lesions. Epinephrin is not a curative drug in the sense that it acts on and neutralizes the poliomyelitic virus. Any favorable effect that it may produce results from its action on the blood-vessels and the consequent control of exudation.

THE NATURE OF THE BACTERICIDAL SUBSTANCE IN LEUCOCYTIC EXTRACT.*

By WILFRED H. MANWARING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

The phenomenon of phagocytosis furnishes evidence of the existence of powerful bacteriolytic substances within the cytoplasm of certain body cells. These endolysins are apparently quite distinct from serum bacteriolysins, since phagocytic cells are capable of destroying many bacteria that are not seriously injured by extracellular body fluids.

There is evidence that, under certain conditions, these intracellular lysins may be given off into the surrounding medium in sufficient quantities to play an important extracellular rôle. The automatic sterilization of old abscess cavities and the sterilization of the pneumonic lung could be accounted for by the assumption of the liberation of such endolysins as a result of cellular disintegration. This has given rise to the hope that eventually a valuable therapeutic agent may be obtained from phagocytic cells. It is conceivable that the therapeutic control of a number of infectious diseases, such as tuberculosis, pneumonia, and the various suppurations, may depend upon a knowledge of these substances. I have therefore undertaken to extend the present knowledge of these lysins, directing my initial effort to a determination of the approximate chemical nature of the bacteriolytic agent that can be extracted from leucocytes.

Considerable work has already been done with leucocytic extract.¹ Most investigators have studied rabbit leucocytes. These they have usually suspended in physiological saline, and have generally

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¹ For a bibliography and resumé of previous work on this subject, see Kling, Carl A., Untersuchungen über die bakterientötenden Eigenschaften der weissen Blutkörperchen, *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 1.

brought about the liberation of the bacteriolysin by repeated freezings and thawings, by heating the suspension to 50° C. for half an hour, or by simply allowing the suspension to undergo autolysis (table I).

TABLE I.

Bacteriolysin from Rabbit Leucocytes.

Twelve discarded rabbits were used as the source of the leucocytes. Each rabbit was injected in the right pleural cavity with 10 c.c. of a 5 per cent. suspension of aleuronat. The resulting pleural exudates were aspirated twenty-four hours later, added to an equal volume of 1.5 per cent. sodium citrate, and immediately centrifugalized. There were thus obtained about 10 c.c. of sediment consisting microscopically of a mixture of red and white cells in the approximate ratio of 1:1. No bacteria were seen, and samples transferred to agar gave no growth.

This sediment was washed twice by centrifugalization with physiological saline, was then suspended in four volumes of distilled water, and placed in the thermostat over night. In the morning the resulting extract was freed from cellular elements by centrifugalization, was heated to 58° C. for thirty minutes to destroy possible traces of serum bacteriolysin, and was then tested in various dilutions for its bactericidal power. In making these tests a loopful of an eighteen hour broth culture of *B. typhosus* was added to 1 c.c. of each fluid to be tested, and plates were made from the resulting mixtures at the times indicated. The table records the number of colonies on the plates thus obtained. The dilutions were made with fifth-physiological saline solution (p/5 sodium chlorid) to preserve osmotic relationships.

Material tested.	Time of plating.				
	1 min.	½ hr.	1¼ hrs.	3 hrs.	24 hrs.
Control, p/5 sodium chlorid	1,080	1,090	1,030	930	940
Undiluted extract	1	0	0	0	0
Diluted extract 1:2	20	0	0	0	3,000
Diluted extract 1:4	720	180	80	50	5,000
Diluted extract 1:8	1,240	1,100	1,200	1,340	1,000

The bactericidal agent thus obtained is relatively thermostable. It can be heated to 60° C. for an hour without loss of bactericidal power, and even to 80° C. without complete inactivation. This furnishes an easy means of differentiating this bacteriolysin from the serum bacteriolysin with which it may be mixed, since most sera are inactivated by heating to 55° C. for half an hour.

In addition to the thermostability, the most suggestive property of the extract for our present purposes is its loss of bactericidal

power on being passed through a Berkefeld filter (table II). From this it has been argued that the bacteriolysin is probably a colloid, presumably proteid in nature. Another suggestive property is the

TABLE II.

Effect of Filtration on Bacteriolysin.

Extract from rabbit leucocytes tested for its bactericidal power before and after its passage through a Berkefeld filter. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Original extract.....	600	0	0	0
Filtered extract.....	1,120	1,020	950	20,000

apparent insolubility of the active principle in ether, which has been taken to indicate that the bacteriolysin is probably not a simple soap or lipoid. The extract can be evaporated to dryness without loss of its bactericidal properties.

The greatest handicap to a more extensive chemical study of the extract has arisen from the small amount of substance with which previous investigators have worked. By injecting aleuronat into the pleural cavity of a rabbit, one seldom obtains more than a single cubic centimeter of leucocytes, which on extraction yields but a few milligrams of dried bacteriolysins. It was thought that this handicap might be overcome by using as the source of material some large animal. The horse was therefore selected for this work.

Rabbit Leucocytes.—As an introduction to the study of horse leucocytes, most of the previous work with rabbit leucocytes was repeated, and the properties outlined above were confirmed. The knowledge of the nature of rabbit leucocytic extract was extended somewhat. Thus, it was shown that for the purpose of the experiment it was immaterial whether the leucocytes were obtained under septic or aseptically conditions. After heating the extracts to 60° C., the extracts obtained under septic conditions and those secured aseptically were apparently identical. Most of the septic extracts, however, if unheated, rapidly lost their bactericidal properties, due presumably to bacterial overgrowth.

The number of red blood corpuscles mixed with the leucocytes

was found to influence the nature of the extract obtained. In the earlier experiments the strongest and most uniform bacteriolysins were obtained from leucocytes mixed with a relatively large number of red cells. So uniform was this relationship, that it suggested the possibility of the bacteriolysin being a product of the red cells, rather than a direct product of the leucocytes. This, however, was shown not to be the case, since equally strong bacteriolysins were subsequently obtained from leucocytes free from red blood corpuscles. The presence of red cells, however, influences the autolytic processes leading to the liberation of the endolysin. The exact nature of this influence has not yet been determined.

In the earlier experiments it was noticed that the leucocytic extracts, freed from cellular elements, usually lost their bactericidal powers on standing. After four or five days in the ice chest, most of the earlier extracts were inactive. It was found that this deterioration could be prevented by heating the extract to 58° C. for thirty minutes. This heating presumably leads to a destruction of proteolytic enzymes responsible for the deterioration.

It was found that the extract could be freed from hemoglobin without loss of bactericidal power by heating it to 62.5° C. for twenty minutes. When so heated, most of the hemoglobin is coagulated, leaving the supernatant fluid nearly colorless.

In working with a substance whose presence or absence must be followed throughout a series of chemical manipulations by tests of bactericidal power, it is necessary to control the osmotic properties at each stage of the manipulation. An osmotic pressure much above that of physiological saline (0.85 per cent. sodium chlorid) is injurious to many bacteria. It was thought that the easiest way to control osmotic relations would be to free the initial extract as completely as possible from crystalloids. This was done by dialysis. It was found that the extract from the rabbit leucocytes could be dialyzed free from sodium chlorid, and presumably free from most of the other crystalloids, without loss of bacteriolytic properties. Celloidin sacs were used as the dialyzing membranes.

Horse Leucocytes.—With this introduction the study of horse leucocytes was begun. These were obtained by intrapleural injec-

tions of aleuronat. It was found advisable in making these injections to observe the following precautions.

1. The material injected should be sterile, and free from irritating substances. The presence of irritating or infectious agents usually causes a rapid pouring out of fluid into the pleural cavity, and extensive diapedesis of red blood cells, but causes the liberation of comparatively few leucocytes.

2. The amount injected should not be too large. Too large an injection causes the formation of a layer of fibrin and aleuronat over the pleural surfaces, through which layer leucocytes apparently have difficulty in making their way. From 300 to 500 cubic centimeters of 5 per cent. aleuronat in 2 per cent. starch paste is a sufficient dose, the starch being added to prevent too rapid sedimentation.

3. The injection should be made in such a manner as to distribute the aleuronat widely throughout the pleural cavity. I usually insert the needle close to the vertebral column, in order that part of the aleuronat may pass to the mediastinal surfaces.

4. The resulting exudate should be aspirated at frequent intervals, to prevent degeneration.

Following such an injection, there are usually formed from one to three liters of pleural exudate daily, for the first week, the daily yield then decreasing and usually ceasing about the fifteenth day. The exudate usually contains about 5 per cent. of leucocytes, often quite free from red blood corpuscles.

The extraction of a bactericidal substance from these leucocytes offers considerable difficulty. Most of the methods which gave successful extracts with rabbit leucocytes were tried, but the extracts obtained were almost uniformly without bactericidal power. One reason for this initial failure is the great variation, not only in the leucocytes of different horses, but also in the leucocytes of the same horse on different days after the aleuronat injection. Horse leucocytes are also apparently very easily injured by manipulation, and easily influenced in their autolytic processes. The exact nature of these factors I hope to make clear in a later paper.

Most of the earlier successful extracts were obtained from un-

washed leucocytes, mixed with comparatively large numbers of red blood corpuscles. This gave rise to the following method of extraction, used throughout this paper.

The pleural exudate, drawn into about a tenth of its volume of 3 per cent. sodium citrate, was enriched by the addition of a sufficient amount of ~~horse~~ citrated horse blood to make the ratio between the red and white cells approximately 1 to 1. The cells were then thrown down by centrifugalization, washed once at 0° C. (to prevent agglutination) with 50 per cent. horse serum, suspended in about four volumes of distilled water, incubated at 35° C. for three hours, and then packed in ice over night. The next morning the resulting extract was freed from cellular elements by centrifugalization, heated to 58° C. for thirty minutes, and tested for its bactericidal power (table III).

TABLE III.

Bactericidal Extracts from Horse Leucocytes.

Eleven extracts from horse leucocytes prepared by the technique described above. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Extract 1	1,520	1,280	1,120	5,000
Extract 2	1,100	880	730	750
Extract 3	980	760	280	550
Extract 4	750	75	10	60
Extract 5	1,000	190	35	0
Extract 6	400	6	2	0
Extract 7	930	128	0	0
Extract 8	580	2	0	0
Extract 9	600	0	0	0
Extract 10	120	0	0	0
Extract 11	4	0	0	0

About two thirds of the extracts prepared by this method were found to possess fairly strong bactericidal properties. The bactericidal power, however, was usually less than that of the extracts from rabbit leucocytes. It is probable that further study of the method of extraction may lead to stronger and more uniform results.

The bactericidal substance in these extracts has apparently the same general properties as the bactericidal substance from rabbit

leucocytes. There are minor differences, as in thermostability and in relation to dialysis, to be presented in detail in the final paper, but the agreement throughout is sufficiently close to warrant the belief that in this extract also the bactericidal agent is probably a proteid. The question then arises as to the class of proteid to which it belongs.

Proteids are usually divided into classes according to their solubility or insolubility in distilled water and in various salt solutions. The process of salting out, in which certain salts are added to a solution in certain concentrations, furnishes an easy means of separating proteids of different classes. This process was applied to the leucocytic extract, but with results that at first were both inconsistent and contradictory and markedly inconstant.

Thus, in one experiment ammonium sulphate was added to the extract to saturation. A heavy precipitate formed immediately. This was thrown down by centrifugalization, washed once with saturated ammonium sulphate, redissolved in a small volume of distilled water, and dialyzed free from ammonium sulphate. The solution thus obtained was evaporated to dryness *in vacuo*, and tested for its bactericidal properties. Making no allowance for amounts lost during manipulation, the end-product was found to possess approximately half the bactericidal power of the initial extract.

This experiment was then repeated, adopting precautions to make the determination an accurate quantitative one. A larger initial volume of extract was taken, the precipitate was dissolved in a larger amount of distilled water, the centrifuge tubes and pipettes were carefully rinsed out and the rinse water was added to the solution to be dialyzed. The end-product of this more careful determination was without bactericidal properties.

Here are two determinations, on superficial examination identical,—one giving a strongly bactericidal end-product, the other an end-product without bactericidal powers. This indicates either that the technique was greatly at fault, or that there are complexities in the determination not sufficiently taken into account in the experimental method.

The only apparent difference between the two experiments is a

volumetric one. As a result of the attempt to make the second determination a quantitative one, the second precipitate was dissolved in a larger volume of distilled water. It was thought that the resulting differences in concentration might possibly make a difference in the subsequent process of dialysis. A study was therefore made of the effect of dilution on dialysis, with the discovery that dilution alone is sufficient to destroy completely the bactericidal properties of the extract within the time necessary for dialysis (table IV).

TABLE IV.

Effect of Dilution on Bacteriolysin.

Sample of a concentrated extract from horse leucocytes diluted with fifty volumes of distilled water and allowed to stand in the ice chest for forty-eight hours. The fluid was then evaporated to dryness and tested for its bactericidal power. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	2 hrs.	5 hrs.	24 hrs.
Diluted extract	920	720	700	1,200
Diluted extract 1: 2	940	820	740	1,100
Diluted extract 1: 4	930	750	770	1,200
Diluted extract 1: 8	900	720	730	800
Diluted extract 1:16	880	830	780	1,000
Original extract	820	110	3	0
Original extract 1: 2	800	30	3	0
Original extract 1: 4	700	40	1	0
Original extract 1: 8	890	610	330	24
Original extract 1:16	900	800	600	340

With this knowledge the experiment was repeated, the method being so modified as to avoid dilution. Instead of dissolving the precipitate in distilled water it was transferred to the dialyzing sac in the solid condition and allowed to go into solution in the small amount of water that entered the sac as a result of the differences in osmotic pressure. In this way it was found possible to dialyze the precipitate free from ammonium sulphate without allowing the volume at any time to increase above the initial volume from which the precipitate was obtained.

The products isolated by this method still show variations, but the results are sufficiently constant to warrant the provisional con-

clusion that the bactericidal substance is probably precipitated quantitatively by full saturation with ammonium sulphate (table V).

TABLE V.

Precipitation with Ammonium Sulphate.

To a sample of the salt-free extract pulverized ammonium sulphate was added to saturation, and the mixture was allowed to stand for one hour at room temperature. The resulting precipitate was thrown down by centrifugalization, washed once with a small amount of standard ammonium sulphate, and pressed between sheets of sterile filter paper to remove as much of the ammonium sulphate solution as possible. The solid precipitate was now transferred to a celloidin sac and dialyzed five times at 4° C. against 1,500 c.c. of double distilled water.

During the dialysis the precipitate went into solution in the small amount of water entering the sac, this volume increasing by the end of the dialysis to about two thirds of the original volume of the extract from which the precipitate was obtained. At the end of the dialysis this volume was made up to the original volume by the addition of distilled water, and the bactericidal power of the resulting solution was compared with that of the original extract. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	2 hrs.	5 hrs.	24 hrs.
Precipitate	580	40	4	0
Precipitate 1: 2	580	20	3	0
Precipitate 1: 4	470	50	2	0
Precipitate 1: 8	1,020	1,120	720	20
Precipitate 1:16	1,010	940	810	550
Original extract	920	60	8	0
Original extract 1: 2	940	20	3	0
Original extract 1: 4	900	30	1	0
Original extract 1: 8	970	350	160	110
Original extract 1:16	1,040	870	770	810

The existence of antibactericidal substances in the extract not precipitated by ammonium sulphate may necessitate a subsequent modification of this conclusion. A study of precipitation by partial saturation with ammonium sulphate and precipitation with other salts is in progress, and will be reported in a later paper.

The earlier attempts to determine the relation of the bactericidal substance to alcoholic precipitation were also confusing. Samples of the extract were added to alcohols of various concentrations, the

resulting precipitates were thrown down by centrifugalization, and the supernatant alcohol decanted and evaporated to dryness. By this means the leucocytic extract was separated into two fractions,—an alcohol-soluble and an alcohol-insoluble part. These two fractions were taken up, either in distilled water or in physiological saline, or usually in some dilution of physiological saline, as fifth-physiological or tenth-physiological, and tested for their bactericidal properties.

In estimating bactericidal power it is desirable to have the osmotic pressures the same in the various parallel fluids to be tested. The dissolving and diluting fluids used were, therefore, those that would make the osmotic pressures equal throughout the determination. The results of these determinations were contradictory and uncertain.

Part of this early confusion I can now attribute to a destruction of the bacteriolysin as a result of dilution, a source of error not suspected at the time the determinations were made. A second source of error was subsequently discovered, the wholly unsuspected antibacteriolytic action of sodium chlorid.

It was early observed that the extracts from horse leucocytes usually increased considerably in bactericidal power on dialysis (table VI). This was at first attributed to the removal of inhibiting proteids as a result of the routine reheating at the end of dialysis, but a subsequent study showed that dialysis alone caused such an increase.

TABLE VI.

Effect of Dialysis on Bacteriolysin.

Samples of extract from horse leucocytes tested before and after dialysis. The dialyzed sample was reheated to insure sterility. Test organism, *B. typhosus*.

	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Before dialysis.....	780	560	330	900
After dialysis.....	550	180	8	0

Sodium chlorid added to a salt-free extract generally decreases its bactericidal power (table VII), the presence of 0.85 per cent.

sodium chlorid often being sufficient to inhibit completely bacteriolysis, while as little as 0.2 per cent. sodium chlorid produces an appreciable lessening. There was, therefore, sufficient sodium chlorid in most of the solvents employed in the earlier tests to render these determinations inconclusive. In repeating the work, distilled water was selected as the dissolving and diluting medium.

TABLE VII.

Effect of Sodium Chlorid on Bacteriolysin.

Samples of a salt-free purified bacteriolysin from horse leucocytes tested for its bactericidal power both alone and in the presence of increasing amounts of sodium chlorid. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Bacteriolysin, water.	790	210	15	0
Bacteriolysin, +0.2 per cent. sodium chlorid.	700	120	25	2,500
Bacteriolysin, +0.4 per cent. sodium chlorid.	1,090	480	90	5,000
Bacteriolysin, +0.85 per cent. sodium chlorid.	1,180	750	660	10,000

If leucocytic extract is added to absolute alcohol, a precipitate forms immediately. If this precipitate is washed with absolute ether and dried *in vacuo*, there is obtained a product soluble in distilled water, possessing about half the bactericidal power of the original extract. If, instead of immediately washing the precipitate with ether, it is washed with absolute alcohol, and is then dried, the final product is largely insoluble. By employing distilled water, however, a fairly strong bactericidal agent can be extracted from this precipitate. This water extract is free from hemoglobin and is presumably free from many other contaminating proteids. It represents the bactericidal agent in the highest degree of purity thus far obtained. The properties of this purified bacteriolysin are now under investigation.

No final conclusion can yet be drawn as to the nature of the bactericidal substance in leucocytic extract. Its behavior toward ammonium sulphate and alcohol are sufficient, however, to warrant the belief that the bactericidal substance is possibly an enzyme. Upon this supposition the investigation is being continued.

CONCLUSIONS.

1. The bactericidal agent extracted from horse leucocytes is apparently precipitated quantitatively by full saturation with ammonium sulphate.
2. The bactericidal agent is apparently precipitated by absolute alcohol, and is not rendered insoluble by a short contact with alcohol. The agent resembled in this feature certain enzymes which can be isolated and purified by alcoholic precipitation.

PURE CULTIVATION OF SPIROCHÆTA PHAGEDENIS
(NEW SPECIES), A SPIRAL ORGANISM FOUND
IN PHAGEDENIC LESIONS ON HUMAN
EXTERNAL GENITALIA.*

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATES 32 AND 33.

In the ulcerative processes situated around the genital region of man one encounters, besides various forms of bacteria, a number of spiral microorganisms belonging to the genus *Spirochæta* or *Treponema*. Thus Schaudinn and Hoffmann¹ described *Spirochæta refringens*; von Prowazek and Hoffmann,² *Spirochæta balanitidis*; Róna,³ *Spirochæta gangrænosa nosocomialis*; Corbus and Harris,⁴ forms resembling Vincent's spirillum; Mulzer,⁵ *Spirochæta pseudo-pallida*; and Polland,⁶ a large spirochæta with five or six blunt curves.

While *Spirochæta refringens* is now known to be non-pathogenic, the etiological relation of *Spirochæta balanitidis* to balanitis erosiva circinata or that of *Spirochæta gangrænosa nosocomialis* to ulcus gangrænosum genitalicum has not been definitely established. Several difficulties must be overcome in order to settle this question.

First of all, it is not easy by morphology alone satisfactorily to identify a spiral organism found in certain lesions with the organisms previously described by these authorities. For example, there

* Received for publication, May 15, 1912.

¹ Schaudinn, F., and Hoffmann, E., *Arch. a. d. k. Gsndhtsamte*, 1905, xxii, 527; *Berl. klin. Wchnschr.*, 1905, xlii, 673.

² Hoffmann, E., and von Prowazek, S., *Centralbl. f. Bakteriöl., Orig.*, 1906, xli, 741, 817.

³ Róna, S., *Verhandl. d. deutsch. dermat. Gesellsch.*, 1907, ix, 471.

⁴ Corbus, B. C., and Harris, F. G., *Jour. Am. Med. Assn.*, 1909, lii, 1474.

⁵ Mulzer, P., *Berl. klin. Wchnschr.*, 1905, xlii, 1144.

⁶ Polland, R., *Wien. klin. Wchnschr.*, 1905, xviii, 1236.

are certain investigators⁷ who fail to recognize any essential morphological differences between *Spirochæta refringens* and *Spirochæta balanitidis*, while others⁸ hold that there are at least four distinct varieties which were indiscriminately described under the name of *Spirochæta refringens*. From this it is easily seen how difficult it is to establish the etiological relation of any one of those organisms to a given definite pathological condition with which it is associated.

The second difficulty is that up to the present we have been unable to isolate these organisms in pure culture and have, therefore, been unable to determine their pathogenicity. The same lack of clearness surrounds the organism of Róna and those of the others. It appears that only a systematic cultural study of all these organisms can clear up the situation and it is for this reason that I describe in this article a spiral organism that has been isolated and grown in pure culture from a rather slowly progressing phagedenic lesion on the external genitalia of a woman.

Material and Cultivation.—The entire left labium showed an enormous swelling with induration, redness, and slight edema. The surface was moist with a seropurulent discharge from the vagina. In the center of the indurated region was an ulcer two by four centimeters and this was covered along its thickened edge with whitish serofibrinous matter. When touched, the ulcer was painful and it bled readily. The lesion had been present for about ten days and was increasing slowly in area. Under local anesthesia a portion of the ulcerated tissue was removed and used for the cultivation experiments.

The tissue thus obtained was rinsed thoroughly in sodium citrate saline solution and then ground in a sterile mortar by adding a fresh lot of citrate solution. In the resulting emulsion, the dark-field microscope revealed numerous irregularly wavy organisms which had a motility of their own that was slow but unmistakable. The length of the spirochætæ varied between four and thirty

⁷ Rille, *München. med. Wchnschr.*, 1905, lii, 1377; Kraus, A., *Arch. f. Dermat. u. Syph.*, 1906, lxxx, 255.

⁸ Eitner, E., *München. med. Wchnschr.*, 1907, liv, 770; Richards, G. M. O., and Hunt, L., *Lancet*, 1906, i, 667.

microns, but was usually about fifteen microns; the width was about 0.75 of a micron. The number of waves varied greatly, some having as many as eight and others only one or two, and some were almost straight. The wave length also varied greatly in different spirochætæ. Very frequently the organism took the form of the interrogation point. The ends were definitely pointed although not sharply drawn out. The short spirochætæ bent their bodies very slowly almost to a semicircle and then stretched out suddenly with a whipping motion. Some forms moved the terminal portion of the body to the left and right very much as an earth worm moves its head. The organisms were stained by Giemsa's stain, but did not retain the Gram stain.

The cultivation experiments with the material described above were carried out by means of a special medium and procedure described by me⁹ for obtaining pure cultures of *Treponema pallidum*, *Treponema mucosum*, and *Spirochæta refringens* directly from contaminated human materials. Briefly stated, a number of ascitic agar tissue tubes were inoculated with the emulsion by means of a sterile capillary pipette. After inoculation the tubes were covered with sterile paraffin oil and incubated at 37° C. After a few days the stab canals of these tubes were filled up with the dense whitish colonies of the bacteria (chiefly staphylococci), but the rest of the agar column remained perfectly clear. At the end of one week some tubes commenced to show a very faint haze at various points along the stab canal, suggesting a colony of the pallidum. The intensity and size of the haze gradually increased, until, after about two weeks, there was an unmistakable growth of some organism. I left the tubes undisturbed for four weeks and then examined them to determine the cause of this hazy appearance. When the hazy portion of the media was taken out with a capillary pipette and examined under the dark-field microscope it showed a mass of rather heavy spiral organisms and a large number of round refractive bodies. Some of the organisms still showed distinct, apparently normal outlines, while others were granular and partly disintegrated. The round bodies were doubtless derived from the breaking up of these partly disintegrated

⁹ Noguchi, H., *Jour. Exper. Med.*, 1912, xv, 90, 466.

forms. Transplants were made at once to a series of fresh culture tubes (ascitic agar tissue medium) in order to purify the culture. After several successive transplants a pure culture of the organism was finally obtained, and during the ten months that have elapsed since its purification the culture has passed through twenty sub-cultures.

Properties of Pure Cultures.—In the ascitic agar tissue medium a faint haze appears around the tissue within forty-eight hours and gradually extends upwards. The density of the growth increases, and within ten days it can be recognized without difficulty (figure 1). The organisms grow at 37° C., but not at 15° C. The strain is still transplantable after having been cultivated for three months at 37° C.

The length of different organisms varies considerably. In young cultures the majority of the spirochætæ measure ten to fifteen microns and show only one or two waves (figures 2, 3, 4, and 5). In old cultures many organisms attain a length of twenty to thirty microns, and the waves are more distinct and numerous (figure 6). The curvature and alternation of the waves are very irregular, and the changes in form are very gradual (figures 7, 8, and 9). The width of the body measures about 0.7 to 0.8 of a micron and is not always uniform throughout the entire length. In a long form there may be seen a nodular swelling and constriction at the middle or at each third of the body. The organisms usually have ends that are fairly pointed, although they may end obtusely. Young forms are frequently straight, but they may bend their bodies into parabolic curves, and then suddenly straighten out again. They also have a sluggish forward movement like that of a creeping worm. The terminal portion of the body is quite flexible and is moved like a feeler. Longer individuals are less motile. There is no flagellum, terminal projection, or undulating membrane. In a very old culture the majority of the organisms become granular and finally disintegrate into small fragments. At the same time there appear a large number of spherical bodies measuring 1.5 microns in diameter. These round bodies are often attached to the ends or the sides of short forms that are still well preserved (figures 10 and 11). When stained with Giemsa's solution these bodies show a small dot

of chromatic material at one pole, but do not take the ordinary bacterial spore stains. Another interesting feature of the organisms is that, under certain undetermined cultural conditions, they produce during disintegration numerous round or ovoid bodies of varying sizes, some as large as three microns in diameter, scattered singly, in pairs, or in clusters (figure 12). At one side of the protoplasmic mass of these bodies a careful examination reveals the presence of a highly refractive spot. In some of the larger forms there are two of these spots. In certain cultures there were transitions between the spherical bodies and those that contain one or two highly refractive spots in their more solid appearing protoplasm. The highly refractive points of the latter take a deep red stain with Giemsa's solution. Forms were also observed which showed more or less irregular refraction in different parts of the spiral body. This was possibly due to cross-bar concentration of the chromatin before the granular segmentation of the organism. In a stained preparation these refractive portions take a deep nuclear stain.

The organism is difficult to stain with most anilin dyes and does not retain the Gram stain. With Giemsa's solution it stains red.

It is a strict anaerobe and requires for its growth the presence of fresh sterile tissue in the ascitic fluid agar (the serum of rabbit or sheep is unsuitable). The protein constituents of the tissue and ascitic fluid are not visibly altered through its growth, but a distinct putrefactive sour odor somewhat resembling butyric acid develops in the culture.

Pathogenicity.—When introduced intradermically into a *Macacus rhesus* monkey and into rabbits an acute inflammation follows within twenty-four hours, but the reaction subsides in about three days without causing an ulcerative process. In the testicles of rabbits it causes a temporary induration which disappears completely within forty-eight hours.

Identification of the Organism.—Since the recent investigations of Gonder,¹⁰ Gross,¹¹ Schellack,¹² Novy,¹³ von Prowazek,¹⁴ Keys-

¹⁰ Gonder, R., *Centralbl. f. Bakteriol., Orig.*, 1909, xlix, 190.

¹¹ Gross, J., *Mitt. a. d. zool. Station z. Neapel*, 1910, xx, 41.

¹² Schellack, C., *Arch. a. d. k. Gsndtsamte*, 1908, xxvii, 364; 1909, xxx, 351.

¹³ Novy, F. G., and Knapp, R. E., *Jour. Infect. Dis.*, 1906, iii, 291.

¹⁴ von Prowazek, S., *Centralbl. f. Bakteriol., Orig.*, 1908, xlii, 229.

selitz,¹⁵ Swellengrebel,¹⁶ and others, the classification of a spiral organism has become considerably more difficult. These authorities have shown that typical spirochætæ may fail to possess all the characteristics formerly regarded as differentiating the spirochætæ from the spirilla; *e. g.*, longitudinal fission, presence of an undulating membrane, absence of flagellum, etc.

In differentiating spirochætæ from spirilla, the importance once attributed to the mode of division has been still further decreased by the discovery of Schmeidlechner,¹⁷ that a group of bacilli multiplies by longitudinal division.

The flexibility of the body was also once considered to be characteristic of spirochætæ, but the organisms of the relapsing fevers (hitherto called spirochætæ or spirilla indiscriminately) seem to have as much flexibility as any of the spirochætæ, and yet on account of the fact that they are supposed to divide transversely, they have been called spirilla by certain authorities (Novy,¹⁸ Fraenkel,¹⁹ Borrel,²⁰ Levaditi,²¹ etc.).

It seems that spirochætæ, and some varieties of spirilla, have certain features that are possessed by the protozoa, and others that are characteristic of the bacteria. They occupy, therefore, an intermediary position between the protozoa and bacteria.

All the various spirochætæ that I have studied have shown features which are more highly differentiated than those seen in bacteria. For example, in most of the spirochætæ we observe during certain periods of their life the secretion of a small round body that stains like chromatin. The organisms often concentrate the chromatin material at one part of the body and then undergo a peculiar segmentation. The granules thus liberated seem to remain alive and at certain periods develop into spiral forms. Again, at

¹⁵ Keysselitz, G., *Arb. a. d. k. Gsndhtsamte*, 1906, xxiii, 566.

¹⁶ Swellengrebel, N. H., *Compt. rend. Soc. de biol.*, 1907, lxii, 213; *Ann. de l'Inst. Pasteur*, 1907, xxi, 448, 562; *Centralbl. f. Bakteriöl., Orig.*, 1909, xlix, 529.

¹⁷ Schmeidlechner, K., *Ztschr. f. Geburtsh. u. Gynäk.*, 1905, lvi, 291.

¹⁸ Novy, F. G., and Knapp, R. E., *loc. cit.*

¹⁹ Fraenkel, C., *Berl. klin. Wchnschr.*, 1907, xlv, 681; *Centralbl. f. Bakteriöl., Orig.*, 1908, xlvii, 471; *München. med. Wchnschr.*, 1907, liv, 201.

²⁰ Borrel, A., *Compt. rend. Soc. de biol.*, 1906, lviii, 138.

²¹ Levaditi, C., *Ann. de l'Inst. Pas'tur*, 1906, xx, 593, 924.

certain stages of their life some of the spirochætæ become banded or dotted with the chromatin substance.

These primitive nuclear phenomena and also the distinct differentiation of the cytoplasm of some species into ectoplasm and entoplasm appear to me sufficiently characteristic to hold these organisms together under the group of spirochætæ without classifying them either with the bacteria or with the protozoa. Upon the basis of the conception just outlined I place my organism among the spirochætæ, but should further investigation make it possible to distinguish between spirochætæ and spirilla, and should it be evident that the organism is wrongly classified I should like to be permitted to correct the error.

Returning now to the question of whether the organism here described has been previously found by other investigators, it appears that the spirochætæ reported by Róna and Polland bear a certain resemblance to my organism and may possibly be identical with it, but I am not justified in concluding that this is the case, and to do so might be misleading; for if my spirochæta is different, I should be giving wrong cultural and biological properties to their organism. In order to avoid such confusion I propose to call my organism *Spirochæta phagedenis*, a conventional name merely indicating its source, but not necessarily showing its etiological relation to the lesion. In the event that this particular organism is found constantly in the phagedenic ulcers on the external genitalia and not in other conditions, its etiological significance would be established. Through lack of material I have up to the present been unable to pursue this subject further.

CONCLUSIONS.

1. A hitherto undescribed spiral organism has been isolated in pure culture from a case of mild phagedenic ulcer on the external genitalia of a woman. For this organism the name *Spirochæta phagedenis* is proposed.

2. *Spirochæta phagedenis* is a strict anaerobe and grows in the presence of fresh tissue in ascitic agar. It produces no apparent change in the media, but a somewhat offensive odor develops in the culture tube.

3. *Spirochæta phagedenis* incites a slight inflammatory reaction in the skin of a *Macacus rhesus* monkey and in the skin and testicles of rabbits.

4. Its etiological relation to the phagedenic lesions on the external genitalia has not yet been determined.

EXPLANATION OF PLATES.

PLATE 32.

FIG. 1. A stab culture of *Spirochæta phagedenis* in ascitic agar tissue medium, three weeks old, at 37° C.

FIGS. 2 and 3. Smears from a pure culture, three weeks old, stained with Giemsa's solution.

PLATE 33.

The dark-field appearance of *Spirochæta phagedenis* from pure cultures.

FIGS. 4 and 5. Young forms with few waves.

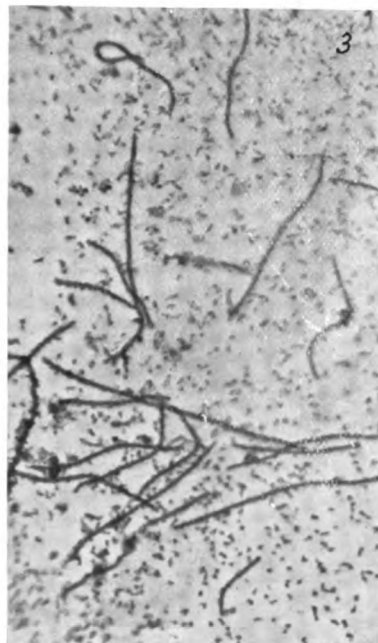
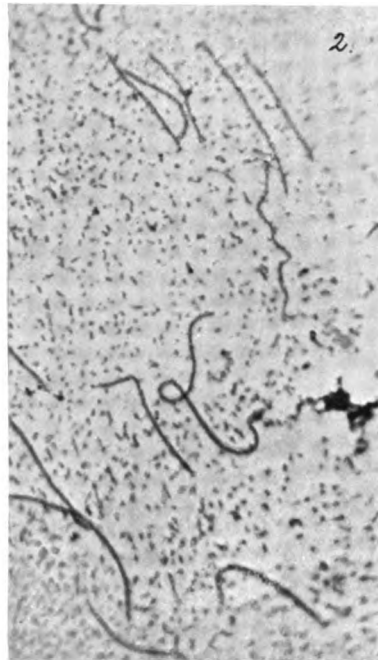
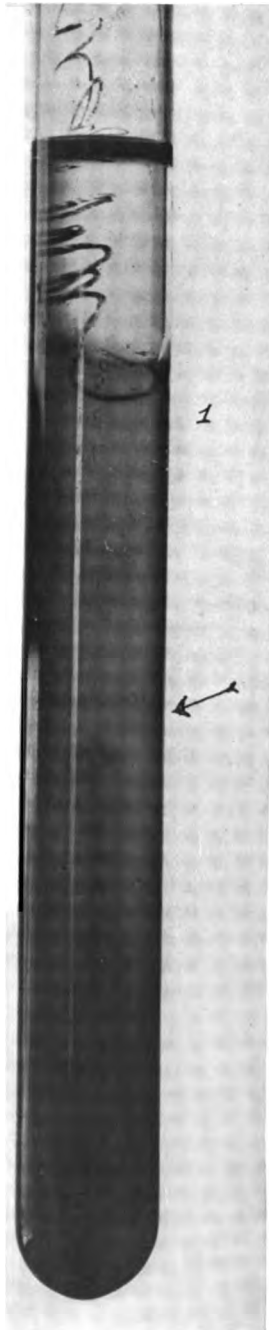
FIG. 6. An average form with shallow, irregular curves.

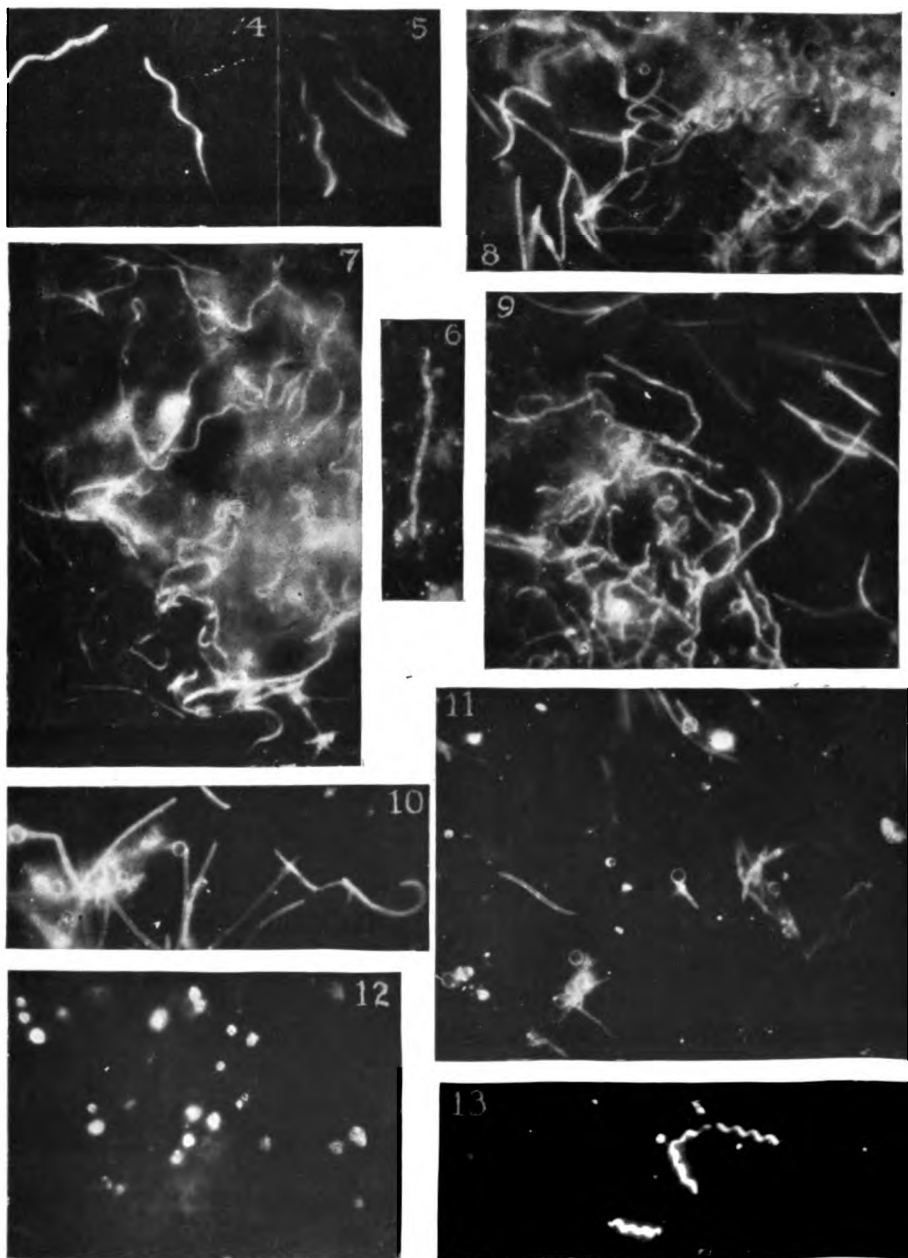
FIGS. 7, 8, and 9. An entangled mass of organisms, with occasional spore-like spherical bodies.

FIGS. 10 and 11. Formation of spherical bodies which are seen still attached to the organisms or already in the free state.

FIG. 12. Plasma granules in an old culture.

FIG. 13. *Spirochæta refringens* from a pure culture (for comparison).





THE DURATION OF IMMUNE BODIES IN THE BLOOD AFTER ANTITYPHOID INOCULATION.*

By MARTHA WOLLSTEIN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Inoculation with dead typhoid bacilli for the prevention of typhoid fever has been successfully practised in the armies of England, Germany, the United States, and Japan. The literature on the subject has recently been reviewed by Major Russell¹ and need, therefore, not be repeated here.

Russell concludes that the incidence of typhoid fever is six to fifteen times as high among unvaccinated soldiers as among those who have been inoculated with the antityphoid vaccine. This clinical immunity to the disease is expressed in the reactions of the serum of inoculated persons to bactericidal, opsonic, and agglutination tests with the typhoid bacillus, the immune bodies appearing in the blood from the fifth to the ninth day after inoculation.

The general impression that immunity after typhoid vaccination lasts about two years seems to be based upon Wright's² original statement that "it is not infrequent to find an agglutinating power in the blood of inoculated persons as long as two years after the inoculation of antityphoid vaccine." Harrison³ found evidence of bactericidal activity higher than normal and of agglutinating power in the serum of one man who had been inoculated four years previously, and in another six years after vaccination. But the reactions were very low, agglutinins in dilutions of 1 to 30 in the former case and 1 to 20 in the latter, while bactericidins were found in dilutions of 1 to 60 and 1 to 40, respectively. Moreover, these two

* Received for publication, May 29, 1912.

¹ Russell, F. F., *Boston Med. and Surg. Jour.*, 1911, clxiv, 1.

² Wright, A. E., *A Short Treatise on Antityphoid Inoculation*, Westminster, 1904, 64.

³ Harrison, W. S., *Jour. Roy. Army Med. Corps*, 1907, viii, 472.

cases had not been followed systematically and their serum reactions to the typhoid bacillus before inoculation are unknown.

Russell⁴ followed "a large number" of cases through weekly blood tests, and found that while agglutinins were sometimes increased on the fourth day, they were always marked by the sixth or eighth day, shooting up rapidly to ten or even twenty thousand. The fall began in about six weeks, and in one case after fifteen months agglutination was present in dilutions of 1 to 80 or 1 to 100. Russell⁵ also found that the phagocytic power of the serum might exceed the normal after a year.

Because of this vagueness in the literature on the duration of immunity after antityphoid inoculation, it seemed worth while to make a systematic study of a definite number of individuals for as prolonged a period of time as seemed necessary. Through the courtesy of Captain H. J. Nichols, of the Army Medical Corps, it was possible to examine the blood of twenty-two soldiers stationed in and around New York City. In addition, the serum of two physicians was studied, making in all twenty-four persons who had received antityphoid inoculations.

The tests were made before vaccination, ten days after each inoculation, and then at intervals over a period of thirteen months. Dr. Nichols very kindly made the inoculations and obtained the blood specimens. Three injections were given to each man, the first of approximately five hundred million dead bacilli, the second and third of one thousand million, at ten day intervals. The vaccine was obtained by Dr. Nichols from the Army Medical School in Washington, and was prepared from a non-virulent strain of typhoid bacillus killed by heating at 60° C. for one hour. One per cent. tricresol was added to the vaccine.

ANIMAL EXPERIMENTS.

Just before this study was begun, Bassenge⁶ had published his observations on the ability of lecithin to increase the immunizing power of the typhoid bacillus, and Wassermann and Seitz⁷ had

⁴ Russell, F. F., *Bull. Johns Hopkins Hosp.*, 1910, xxi, 83.

⁵ Russell, F. F., *Bull. Johns Hopkins Hosp.*, *loc. cit.*

⁶ Bassenge, R., *Deutsch. med. Wchnschr.*, 1908, xxxiv, 139.

⁷ Wassermann, M., and Seitz, A. M., *Deutsch. med. Wchnschr.*, 1908, xxxiv, 2175.

failed to confirm the statements of Bassenge, their experiments leading them to the conclusion that lecithin causes only a local increase in resistance which is short in duration and absolutely non-specific in character. Our preliminary experiments with white rats were not encouraging, since vaccine plus 1 per cent. lecithin produced a serum that agglutinated typhoid bacilli in dilutions of 1 to 2,500, and was bactericidal in a dilution of 1 to 40,000, while a similar dose without lecithin developed a lytic action that was twice as strong. Again, lecithin in 10 per cent. solution did not kill the typhoid bacillus in forty-eight hours, an observation which was confirmed by Vallet and Rimbaud,⁸ who found the bacilli viable after three days' contact with 10 per cent. lecithin. Nor did they find that the addition of a lecithin emulsion to typhoid bacilli produced a serum whose agglutinating power was superior to that obtained from the bacilli alone. It was possible, however, that lecithin might lessen the local reaction, always more or less painful after an antityphoid vaccination, and the experiment of combining lecithin and vaccine was tried on a monkey.

Experiment 1.—A rhesus monkey was inoculated subcutaneously into the right thigh with 1 c.c. of vaccine, which equalled one agar slant of typhoid bacilli killed with 0.04 per cent. lysol. The temperature before inoculation was 40.6° C., and was still the same after two hours. The serum of this monkey did not agglutinate bacilli in a dilution of 1:20, and was bactericidal in dilutions of 1:80.

After twenty-four hours the temperature of the animal was 40.2° C. The thigh was held stiffly and there was a small amount of induration about the point of inoculation. There was no redness. The animal was lively. Next day the temperature was the same and the animal used the leg well. There was no tenderness. Thereafter the monkey seemed perfectly well, and the induration disappeared.

Six days after inoculation the blood agglutinated the bacilli in dilutions of 1:40, but gave a positive bactericidal effect in dilutions of 1:5,120. On the eighth day the animal was reinoculated with double the first dose of vaccine, after which the temperature did not rise, and no pain, tenderness, or swelling was apparent in the thigh. Six days later agglutinins were present in dilutions of 1:640, and bactericidins in dilutions of 1:5,120.

Experiment 2.—A rhesus monkey was inoculated subcutaneously into the right thigh with 1 c.c. of vaccine plus 1 c.c. of 1 per cent. lecithin. The temperature before inoculation was 40.6° C. The serum did not agglutinate the typhoid bacilli in a dilution of 1:20, but killed them in dilutions of 1:80. The fol-

⁸ Vallet, G., and Rimbaud, L., *Compt. rend. Soc. de biol.*, 1910, lxxviii, 302.

lowing day the temperature had not risen; the thigh felt warm and indurated, but it was not tender. The monkey held it stiffly for two days. The temperature rose to 41° C. on the second day; then fell to 40.5° C. on the third day. The induration was gone on the third day. After six days agglutinins were present in dilutions of 1:20, and bactericidins in dilutions of 1:40,960. After a second dose, double the first in size, the monkey remained perfectly well, and no redness, pain, or swelling appeared. After six days the blood agglutinated the typhoid bacilli in dilutions of 1:2,560, and killed the organisms in dilutions of 1:367,680.

The addition of lecithin to the vaccine did not influence the local reaction in this monkey, but it must be admitted that in both experiments the local signs were very slightly marked. Immune bodies were present in higher dilutions in the serum of the animal to which lecithin had been given. On repeating these two experiments, the local reactions were practically similar to those in the first set, but the immune bodies were slightly less marked in the case of the monkey that received lecithin with the vaccine. The question of individual variation of monkeys to typhoid infection evidently enters largely into these results, and lecithin would seem to be inconstant in its action, if it has any at all, as a promoter of typhoid antibody formation.

Since the subcutaneous injection of a 1 per cent. lecithin emulsion, either alone or with antityphoid vaccine, had had no bad effects in rats or monkeys, and since there was still a slight possibility that lecithin added to the vaccine might help to increase the production of agglutinins and bactericidins, it was decided to divide the twenty-four human cases into two groups of twelve; one group was to receive antityphoid vaccine alone, while the other was to receive vaccine plus a 1 per cent. lecithin emulsion. It may be said at once that the lecithin did not, in any case, lessen the pain or the duration of the local reaction, nor, on the other hand, did it in a single instance increase the local signs.

SERUM REACTIONS IN THE INOCULATED PERSONS.

The immunity to typhoid fever depends apparently upon bacteriolytic antibodies in the blood; hence it follows that tests for bacteriolytins or bactericidins are of the greatest importance in judging whether or not a given serum is immune to the typhoid bacillus.

Whether or not the presence of agglutinins adds to the protective power of such a serum we do not know, but it is certain that bactericidins and agglutinins in antityphoid sera do not always run in parallel lines.

In studying the sera from the twenty-four vaccinated persons, bactericidal tests were made according to the method of Stern and Korte,⁹ and agglutinins were also looked for. Phagocytic tests were not made. Russell¹⁰ believes that bactericidal tests are valueless because of the variability of the same strain of the typhoid bacillus to bactericidal action. The strain that we used throughout this work was one that had given good results with preliminary tests on antityphoid goat and rabbit sera. In order to eliminate errors as far as possible, it was our custom to make a test with goat or rabbit serum before examining each set of the human specimens. Schlemmer¹¹ has recently found that some strains of typhoid bacillus are very resistant to the action of bactericidal sera, others less so, but that the degree of serum resistance remains constant for a single strain.

Before the inoculations were begun, preliminary blood tests were made in eighteen cases. Sixteen were negative both as regards agglutinins and bactericidins. One soldier gave a negative agglutination test but a positive bactericidal reaction in a dilution of 1 to 20 only. The serum of another man agglutinated the typhoid bacillus in a dilution of 1 to 40, and killed the organism even when diluted three hundred times. The latter soldier had had typhoid fever three years before. Three other men had previously had a typhoid attack, but their sera were quite devoid of antibodies.

After the first inoculation the lecithin vaccines seemed to give results which were slightly inferior to those produced by the simple suspensions of dead bacilli, four cases giving negative tests with lecithin as against three cases without lecithin. The highest bactericidal titer was one in ten thousand. Only four persons to whom lecithin was given reacted in a dilution of 1 to 300 or over, while of those to whom no lecithin had been given five reacted in dilutions of 1 to 600 and more.

⁹ Stern, R., and Korte, W., *Berl. klin. Wchnschr.*, 1904, xli, 213.

¹⁰ Russell, F. F., *Bull. Johns Hopkins Hosp.*, *loc. cit.*

¹¹ Schlemmer, *Ztschr. f. Immunitätsforsch., Orig.*, 1911, ix, 149.

Ten days after the second dose had been administered there were still three persons with sera having no bactericidins, and each of these persons had received the lecithinated vaccine. Two of these sera, however, contained agglutinins in dilution of 1 to 160 and 1 to 640, respectively. Seven of the sera of persons injected with vaccines containing lecithin killed typhoid bacilli in dilutions of 1 to 5,000 and over, while ten sera of persons vaccinated without lecithin reached as high a titer.

The third inoculation with lecithin raised one of the negative cases to a bactericidal titer of 1 to 40,000, while the other two still remained negative. One serum was bactericidal when diluted a million times, the highest point reached by any specimen. It was obtained from a physician who had had typhoid fever eleven years before the vaccination.

One month after the third inoculation, which was two months after the vaccinations were begun, the immune bodies were present in the sera in the highest amount and thereafter steadily decreased, so that four months after the first inoculation only one specimen reached the 5,000 mark, and that came from a man to whom lecithin vaccine had been given.

After eight months one serum still showed a bactericidal titer of 1 to 2,500, and it came from a soldier to whom lecithin had been administered.

After ten months only fourteen specimens were available for study, as some of the men had been sent elsewhere for duty. Of the fourteen sera, one from a man to whom no lecithin had been given reached a dilution of 1 to 160, while six other sera, four from men who had received lecithin and two from those who had not, failed to react at all.

After thirteen months fourteen sera were again obtained, one of which was bactericidal in a dilution of 1 to 1,280. Ten were negative, two reacted in a dilution of 1 to 40, and one in a dilution of 1 to 80.

Agglutinins dropped out even earlier than did the bactericidins. After four months only two sera agglutinated the typhoid bacillus in a dilution of 1 to 160, and no agglutinins were present in any one

of the fourteen specimens examined thirteen months after vaccination.

DISCUSSION OF RESULTS OBTAINED.

The average bactericidal curve of the antityphoid immune serum resulting from inoculation with dead typhoid bacilli in this series of cases would seem to have been as follows.

Starting from a negative preliminary reaction, the bactericidins appeared after the first inoculation in dilutions of 1 to 80 to 1 to 2,500, with an exceptional dilution of 1 to 10,000; they rose to 1 to 5,000 or 1 to 20,000 after the second dose, remained there or reached 1 to 40,000 after the third inoculation, and remained at that point or rose, exceptionally, to 1 to 80,000 during the following month. Then, two months after the first inoculation, the highest point was reached and the drop began. This was apt to be abrupt and, as a rule, it went below 1 to 5,000, sometimes even to 1 to 160. After four months a titer of 1 to 2,000 was occasionally present, but the majority of sera fell below 1 to 300 and some (four) had already become negative. Eight months after inoculation the conditions were about the same, but after ten months no bactericidal effect was obtained in any dilution above 1 to 160; the majority lay between 1 to 40 and 1 to 80, and at this time more were negative. At the end of thirteen months the great majority of cases gave an entirely negative bactericidal test (ten out of fourteen); a titer of 1 to 40 or 1 to 80 was sometimes noted, and 1 to 1,280 was the single exception. In this last instance lecithinated vaccine had been administered.

Comparing the results from vaccines with and without lecithin, we find that the examples without lecithin developed bactericidins a little more rapidly, but in no greater quantity than the others, while the examples with lecithin seemed to retain the height slightly better. Two months after the first inoculation ten sera from persons vaccinated with lecithin as against eight vaccinated without it reacted in dilutions above ten thousand. The drop in activity was about as rapid in one case as in the other; e. g., five lecithin cases against four inoculated without lecithin reacted in dilutions greater than 1 to 2,000 at the end of three months. But after four months

three negative cases would seem to show that the sera after lecithin vaccination retained their immune bodies for a shorter period.

At the end of thirteen months nine of the twelve lecithin cases had become negative; one reacted in a dilution of 1 to 80, another in a dilution of 1 to 1,200, and the third could not be tested. It had not been available for study since four months after the beginning of the inoculations, and its low titer of 1 to 160 at that time argued strongly in favor of its being negative later.

The sera obtained from the soldiers inoculated without the addition of lecithin gave no negative bactericidal reactions until ten months after the first inoculations, when two cases failed to kill typhoid bacilli in any dilution, and three months later four other cases were negative. The other two sera examined at this time gave positive results in dilutions of 1 to 40 and 1 to 80 only, and the four cases not available at that time had been dropping steadily since their last examination (tables I and II).

A word remains to be said about the four persons in the series who had suffered attacks of typhoid fever from three to fifteen years before being inoculated. The serum of only one, the most recent case, gave a positive preliminary bactericidal test. This serum rose to a titer of 1 to 10,000 after the first dose of vaccine and reached its highest point, 1 to 40,960, two months later. It then dropped gradually, reaching its original strength in eight months, and after thirteen months was negative. Whether this serum would have fallen to zero in that time if the man had not been vaccinated, is a question. Another man, inoculated eleven years after an attack of typhoid, developed bactericidins which were active in dilutions of 1 to 10,000 after the first vaccination, 1 to 40,000 after the second, and 1 to 1,000,000 after the third. They fell to 1 to 10,000 in another month, remaining at 1 to 5,000 for two months and then gradually falling to 1 to 80, still reacting in that dilution fifteen months after the initial inoculation.

This instance, more than any other of the four under discussion, illustrates the point that when the human organism has once been infected with the typhoid bacillus or its toxin, reinoculation more readily results in the formation of immune bodies to that bacillus.

TABLE I.
Antityphoid Vaccination

No. of case.	Preliminary test.		10 days after 1st vaccination.		10 days after 2d vaccination.		10 days after 3d vaccination.		2 mos. after 1st vaccination.	
	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.
1	0	0	1:80	0	1:10,240	1:320	1:10,240	1:320	1:40,960	1:640
2	0	0	1:80	1:80	1:20,480	1:2,560	1:40,960	1:2,560	1:40,960	1:640
3 ¹²	0	0	1:80	0	1:320	1:320	1:640	1:640	1:163,840	1:1,280
4	0	0	0	0	0	Not made	0	0	1:1,280	1:20
5	0	0	1:320	Not made	1:10,240	Not made	1:20,480	1:80	1:40,960	1:80
6	0	0	1:320	Not made	1:10,240	1:320	1:40,960	1:640	1:40,960	1:20
7 ¹²	0	0	1:80	1:320	1:5,120	1:640	1:5,120	1:640	1:40,960	0
8	0	0	0	1:80	0	1:160	1:40,960	1:320	1:40,960	1:320
9	0	0	0	1:40	1:20,480	Not made	1:40,960	1:1,280	1:40,960	1:80
10	0	0	1:10,240	1:40	1:2,560	1:640	1:5,120	1:20	1:40,960	1:640
11 ¹²	0	0	1:10,240	1:80	1:40,960	1:320	1:1,210,720	1:80	1:10,240	1:80
12	1:20	0	0	1:640	0	1:640	0	1:640	1:1,280	1:160

TABLE II.
Antityphoid Vaccination

No. of case.	Preliminary test.		10 days after 1st vaccination.		10 days after 2d vaccination.		10 days after 3d vaccination.		2 mos. after 1st vaccination.	
	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.	Bactericidal.	Agglutination.
1	0	0	1:160	1:160	1:10,240	Not made	1:10,240	1:320	1:20,480	1:640
2	0	0	1:80	1:20	1:5,120	1:640	1:5,120	1:640	1:40,960	1:640
3	0	0	1:640	1:40	1:20,480	1:640	1:20,480	1:640	1:81,920	1:640
4	0	0	1:2,560	1:20	1:20,480	1:320	1:40,960	1:640	1:10,240	1:80
5	Not made	0	0	1:20	1:20,480	1:2,560	1:20,480	1:1,280	1:20,480	Not made
6 ¹²	1:320	1:40	1:10,240	1:80	1:10,240	1:80	1:10,240	1:80	1:40,960	1:1,280
7	Not made	0	0	1:640	1:20,480	Not made	1:20,480	1:320	1:40,960	1:320
8	Not made	0	1:80	1:160	1:40,960	1:320	1:40,960	1:640	1:40,960	1:640
9	Not made	0	1:640	1:160	1:5,120	1:320	1:2,560	1:1,280	1:40,960	1:160
10	Not made	0	0	1:640	Not made	0	1:20,480	1:640	1:80	1:80
11	Not made	0	1:2,560	Not made	1:20,480	1:640	1:20,480	1:640	1:160	1:320
12	0	0	Not made	0	Not made	0	1:20,240	1:640	1:640	1:80

¹² Nos. 3, 7, and 11 had had typhoid fever.

¹² No. 6 had had typhoid fever.

with Lecithin.

3 mos. after 1st vaccination.		4 mos. after 1st vaccination.		8 mos. after 1st vaccination.		10 mos. after 1st vaccination.		13 mos. after 1st vaccination.	
Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.
1:2,560	1:640	1:160	0	1:160	1:80	1:40	1:20	0	0
1:320	1:320	1:80	Not made	1:160	1:20	0	0	0	0
1:640	0	1:80	Not made	Not made		0	0	Not made.	
1:320	1:20	0	0	Not made		Not made		Not made.	
1:1,280	1:80	1:40	0	Not made		Not made		0	
1:80	1:80	0	Not made	Not made		Not made		Not made.	
1:10,240	Not made	0	Not made	1:80	0	1:40	0	0	0
1:5,120	Not made	1:1,280	Not made	1:1,280	1:80	Not made		1:1,280	0
1:1,280	Not made	1:640	1:80	0	0	0	0	0	0
1:40,960	Not made	1:80	1:20	1:320	Not made	0	0	Not made.	
1:5,120	1:20	1:5,120	0	1:320	0	1:80	0	1:80	0
1:640	0	1:160	0	Not made		Not made		Not made.	

thout Lecithin.

3 mos. after 1st vaccination.		4 mos. after 1st vaccination.		8 mos. after 1st vaccination.		10 mos. after 1st vaccination.		13 mos. after 1st vaccination.	
Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.	Bacteri- cidal.	Aggluti- nation.
1:1,280	1:80	1:80	1:40	1:80	1:40	Not made		Not made	
1:320	1:160	1:320	1:160	1:160	1:80	1:40	0	0	0
Not made		1:2,560	1:80	Not made		Not made		Not made	
1:20,480	1:160	1:1,280	1:40	1:640	1:40	1:80	0	0	0
1:20,480	1:160	1:1,280	Not made	1:640	1:80	1:40	1:20	1:40	0
1:1,280	Not made	1:640	1:40	1:320	1:20	1:80	0	0	0
Not made		Not made		1:640	0	0	0	0	0
1:5,120	1:320	1:2,560	1:80	1:2,560	0	1:160	0	1:80	0
1:640	1:160	1:320	Not made	1:320	0	0	0	Not made	
1:1,280	1:80	1:640	1:20	Not made		Not made		Not made	
1:320	1:640	1:320	1:20	Not made		Not made		Not made	
1:40,960	1:80	1:20	1:160	Not made		Not made		0	0

Cole¹⁴ proved this point experimentally in animals, and it is the principle upon which the value of typhoid vaccination rests.

It should be remarked that besides these two men, both of whom had suffered from typhoid fever, only one other reacted in a dilution of 1 to 10,000 after the first dose of vaccine. The serum of the third man who had had typhoid reacted slowly. It was active only in a dilution of 1 to 80 after the first dose, but two months later reached a titer of 1 to 160,000, then dropped gradually in ten months to zero. The strength of the serum of the fourth man rose very slowly until it was active in a dilution of 1 to 40,000, and then in four months dropped rapidly to zero. The attack of typhoid fever in this instance had occurred about fifteen years before.

An exceptionally slow and low reaction was obtained in one soldier whose serum failed to show any bactericidins or agglutinins even after the third inoculation of vaccine. One month later bactericidins were present in dilutions of 1 to 1,200 only, and agglutinins in a dilution of 1 to 20. Three months after the initial dose had been given, the reaction was positive in a dilution of 1 to 300, and after four months it had entirely disappeared. The serum of another man failed to respond to the first and second doses, but reached a titer of 1 to 40,000 after the third dose, and proved to be the only one that contained bactericidins in dilutions of 1 to 1,200 thirteen months later.

SUMMARY.

In this series of twenty-four persons inoculated with antityphoid vaccine, the immune bodies in the blood reached their height within two months after the first inoculation, or one month after the third, then fell rapidly within the next two months. Only nineteen of the cases could be followed longer, and eight of these were negative for bactericidins within ten months after inoculation, and fifteen were negative after thirteen months. Only one serum reacted in a dilution of 1 to 1,200 at the end of thirteen months.

The addition of lecithin to the vaccine did not influence the local reaction after inoculation, nor did it appreciably affect the formation of immune bodies to the typhoid bacillus.

¹⁴ Cole, R., *Ztschr. f. Hyg.*, 1904, xlv, 371.

CONCLUSIONS.

There is, of course, no justification for the conclusion that clinical immunity can be determined absolutely by the measure of immune bodies in the blood, since experience has apparently proved the contrary, a fact which can be accounted for by the latent power of the body cells to react more quickly to a stimulus which has once made them sensitive. That this power lasts for many years after an attack of typhoid fever is well known. Further study is required to prove its duration after antityphoid inoculation. It would seem, however, that reinoculation with typhoid vaccine within a year is indicated when exposure to typhoid fever seems imminent.

THE RETENTION OF FOREIGN PROTEIN BY THE KIDNEY.

A STUDY IN ANAPHYLAXIS.*

By RICHARD M. PEARCE, M.D.

(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.)

This investigation was undertaken for the purpose of obtaining exact information in regard to two points of prime importance in connection with the physiology and the pathology of the kidney: (1) does a foreign protein tend to accumulate in the kidney; and (2) if so, is this tendency greater in the presence of a nephritis? The first problem is of importance mainly in connection with the etiology of nephritis; the second, in connection with the prognosis, treatment, and dietetics of nephritis.

It is obvious that the acute nephritides associated with acute infectious diseases (scarlet fever, diphtheria, etc.) are due either to the poison elaborated by the causal microörganism or to the products of tissue catabolism. Soluble bacterial and protozoan poisons as well as the poisonous products of tissue destruction act on the kidney cells, either in a specific selective way or through irritation as the result of long continued contact during a process of gradual elimination.

The latter has quite generally been assumed to be the correct explanation, but this assumption has no experimental or other data as a basis. Because the glomerulus is the usual seat of the renal lesion of scarlet fever, it is believed that the glomerulus is affected as the result of the elimination by the kidney of the toxins of scarlet fever. So also in the case of cholera and eclampsia, the epithelial lesion in the kidney tubules is supposed to be due to the elimination of the toxins of these diseases through the cells of the

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tubules. Likewise, in the experimental nephritides, the glomerular lesion due to arsenic and the tubular lesions due to potassium chromate are similarly explained.

On the other hand, it is possible that these various poisons may have a specific selective affinity for certain constituents of the various cells of the kidney, and may be "fixed" as it were in these cells, remaining within them appreciably longer than the period usually required for mere elimination.

The solution of this problem depends, therefore, on some method capable of demonstrating that a foreign protein introduced into the blood-stream is (1) equally distributed in the blood and organs; or (2) that it is present in greater amount in the kidney than in the blood; or (3) that it may be demonstrated in the kidney after it has disappeared from the circulating blood. These various possibilities may be tested by a method that is dependent on the phenomenon of anaphylaxis, and the suggestion that these problems could be solved by this method I owe to the papers of Vaughan and his associates¹ on the parenteral introduction of protein, and to the statement of these investigators that a foreign protein (egg-white) may be detected in the kidney of the rabbit after it has disappeared from the circulating blood.

Previously Wells had used the anaphylaxis reaction in the study of alimentary albuminuria, and more recently Van Alstyne and Grant have used it to demonstrate the absorption of albumin from the intestine without digestion. Wells found that guinea pigs can not be sensitized to egg-albumin with the urine of individuals receiving a diet of raw eggs, even when such individuals have an alimentary albuminuria, and concluded that ingested egg-albumin does not pass through the body unchanged, and that the albuminuria under such circumstances is due to the elimination, not of ingested foreign protein, but of serum proteins.

The use of the anaphylaxis reaction to detect foreign protein in the circulating blood or in the organs has been attempted, so far as

¹ Vaughan, V. C., Cumming, J. G., and McGlumphy, C. B., The Parenteral Introduction of Proteins, *Ztschr. f. Immunitätsforsch., Orig.*, 1911, ix, 16; Vaughan, V. C., Cumming, J. G., and Wright, J. H., Protein Fever, *ibid.*, 458.

I am aware, only by Vaughan and his associates, and by Van Alstyne and Grant.²

The latter injected into a vein of a dog 20 c.c. of a mixture of equal parts of egg-albumin and salt solution, and drew blood after one quarter of an hour, and after four, forty-eight, and seventy-two hours. The serum of each of the various samples and the urine collected during the first twenty-four hours were used to sensitize guinea pigs. At a later period an intoxicating injection of egg-albumin caused in each animal the characteristic symptoms of anaphylaxis, these being least marked in the animal sensitized with the serum drawn after seventy-two hours, only moderately severe in the animal receiving the forty-eight hour serum, but severe in all of the others, including the animal that received the urine.

In a second series of experiments Thiry-Vella fistulas were made at various levels of the intestinal canal, egg-albumin was placed in these, and blood was drawn at periods varying from one to three hours. The blood serum thus obtained was injected into guinea pigs and sensitized these animals to egg-albumin. Similar results were obtained with the urine of the first twenty-four hours.

The experiments of Vaughan and his associates may be divided into two groups. In the earlier experiments they used for the sensitization of guinea pigs the blood of rabbits obtained a few hours after the feeding or injection of egg-albumin.

In one experiment they found that one and a half hours after the intravenous injection of egg-albumin (50 c.c. of 1:1 solution) the blood failed to sensitize guinea pigs; in a second, failure was noted after two hours; and in a third, after two and a half hours.

In a fourth experiment blood drawn one hour after the injection of egg-albumin sensitized, while that drawn after two, three, and five hours failed to sensitize; on the other hand, all the extracts of brain, kidney, spleen, and liver, made six hours after the injection of egg-white, possessed a sensitizing power which was most marked in the extracts of the kidney and liver.

In a fifth experiment after the egg-white had disappeared from the circulating blood the animal was transfused with one liter of salt solution and samples of fluid were taken from the heart in amounts of 2 c.c. These had the power to sensitize as did also extracts of liver, spleen, and muscle; extracts of brain and kidney failed to sensitize.

In a later communication it was shown that extracts of skin, kidney, brain, liver, spleen, intestine, and stomach from rabbits killed twenty-four and forty-eight hours after intravenous injection of egg-albumin are capable of sensitizing guinea pigs, but that similar extracts from animals killed after seventy-two hours do not have this power. The symptoms of anaphylaxis are, the writers note, most marked in guinea pigs receiving extracts of kidney and spleen.

It was this difference between the results at forty-eight and seventy-two hours and the marked reaction in guinea pigs receiv-

² Van Alstyne, E. V. N., and Grant, P. A., The Absorption of Albumin without Digestion, *Jour. Med. Research*, 1911, xxv, 399.

ing extracts of the kidney that suggested the use of this method of sensitization for the purposes of the present investigation.

METHODS.

The usual procedure has been to inject intravenously a group of three, four, or five rabbits with a foreign protein, to chloroform the animals at intervals of twenty-four hours, and then to inject their blood or the extracts of their organs into the peritoneal cavity of guinea pigs. After two or three weeks these guinea pigs received either intravenously or intraperitoneally an injection of the same kind of protein originally injected into the rabbits.

Egg-albumin and horse serum have been the substances used in most of the experiments. The egg-white has been diluted with three parts of physiological salt solution, except in some of the earlier experiments in which a dilution of equal parts was tried; but as this dilution frequently caused sudden death, it was abandoned for the dilution of one part to three. With the latter dilution no immediate ill effects have been seen, though very rarely an animal died on the second or third day after the injection.

All injections of egg-white and serum were made in the ear vein, forty cubic centimeters of diluted egg-albumin (ten cubic centimeters of egg-white) and ten cubic centimeters of undiluted horse serum being the amounts injected. All animals were killed by chloroform except when it was necessary to obtain the blood or to wash the organs with salt solution. Under the latter circumstances, ether anesthesia was always used. Extracts were made by grinding the organs in a mortar with sand, adding twenty cubic centimeters of salt solution, and allowing the mixture to stand at 33° to 35° C. for eighteen to twenty-four hours. The fluid was then removed by centrifugalization. The extracts thus obtained were injected into guinea pigs in amounts of five to eight cubic centimeters, and defibrinated blood in amounts of five cubic centimeters. All extracts for sensitization were injected intraperitoneally.

The intoxicating dose was given in the early experiments both intraperitoneally and intravenously; in the later work, injections were given into the jugular vein only. The amount of egg-white used for an intraperitoneal injection was five cubic centimeters of a 50 per cent. (1 to 1) dilution, and for intravenous injection four

cubic centimeters of a 25 per cent. (1 to 3) dilution. Horse serum was given by these two methods in doses of five cubic centimeters and one to two cubic centimeters respectively.

The varying degrees of sensitization, as presented in the tables, are indicated by the following signs:

- ++++ = acute fulminant anaphylaxis with death and typically insuflated lungs at autopsy.
 +++ = marked symptoms,—severe respiratory distress, “bucking,” convulsions, and prostration, but with eventual recovery.
 ++ = moderate attack,—spasmodic cough, respiratory distress, “bucking,” and a tendency to lie prone.
 + = “scratching,” cough, slight “bucking,” slight respiratory distress.
 o = no effect.

The following table confirms Vaughan's single observation as to the presence of egg-albumin in the kidney twenty-four and forty-eight hours after injection, and its absence (or failure to sensitize) after seventy-two hours; it also shows the results of control experiments.

SERIES I.

Egg-White.

The rabbits were killed one, two, three, and four days after intravenous injection of 10 c.c. of egg-white diluted with salt solution. Guinea pigs received intraperitoneally extracts of the kidneys of each rabbit, and after three weeks had elapsed they received intraperitoneally an intoxicating dose of 5 c.c. of a mixture of equal parts of egg-white and salt solution, or intravenously 4 c.c. of a 1:3 dilution.

No. of animal.	Extract.	Method of injection.	Result.
Guinea pig 1	24 hrs.	Intraperitoneal.	+++
Guinea pig 2	24 hrs.	Intravenous.	++++
Guinea pig 3	48 hrs.	Intraperitoneal.	+
Guinea pig 4	48 hrs.	Intravenous.	++
Guinea pig 5	72 hrs.	Intraperitoneal.	o
Guinea pig 6	72 hrs.	Intravenous.	o
Guinea pig 7	96 hrs.	Intraperitoneal.	o
Guinea pig 8	96 hrs.	Intravenous.	o
Control 1 ^a		Intraperitoneal.	o
Control 1		Intravenous.	o
Control 2 ^a		Intraperitoneal.	o
Control 2		Intravenous.	o
Control 3 ^a		Intravenous.	++++

^a Control 1. Normal guinea pigs.

Control 2. Guinea pigs that had received, twenty-eight days before, extracts of normal rabbit kidney.

Control 3. A guinea pig that had received, three weeks before, an extract prepared from normal rabbit kidney and 1 c.c. of egg-white.

SERIES II.

Horse Serum.

This series is like series I except that horse serum was used instead of egg-white. The rabbits were killed one, two, three, and four days after receiving intravenously 10 c.c. of normal horse serum. Extracts of the kidneys of each animal were injected intraperitoneally into guinea pigs, and after three weeks each guinea pig received intravenously 2 c.c. of the same horse serum, or 5 c.c. intraperitoneally.

No. of animal.	Extract.	Method of injection.	Result.
Guinea pig 1	24 hrs.	Intraperitoneal.	+++
Guinea pig 2	24 hrs.	Intravenous.	++++
Guinea pig 3	48 hrs.	Intraperitoneal.	++
Guinea pig 4	48 hrs.	Intravenous.	+++
Guinea pig 5	72 hrs.	Intraperitoneal.	++
Guinea pig 6	72 hrs.	Intravenous.	++++
Guinea pig 7	96 hrs.	Intravenous.	o
Control 1 ⁴		Intravenous.	o
Control 2 ⁴		Intravenous.	o
Control 3 ⁴		Intravenous.	++++

In series II the kidney extract of the third day still retained power to sensitize.

At this point, it seemed desirable to determine whether bacterial proteins were retained by the kidney in a form capable of sensitizing guinea pigs. The work of others on sensitization to tuberculin and to pneumococcus protein offered some hope of successful results, and, moreover, the use of bacterial proteins, which are factors in the production of nephritis, would, it was obvious, allow more definite conclusions concerning their possible retention in man, than would studies with egg-albumin and horse serum.

Rabbits were therefore given intravenously tuberculin in the form of O. T. bovine,⁵ a tuberculin⁶ prepared without heating, mallein,⁷ mallease,⁸ and pneumococcus protein.⁹ A vegetable protein, gliadin,¹⁰ was also used.

⁴Control 1. Normal guinea pig.

Control 2. A guinea pig that had received, three weeks before, an extract of normal rabbit kidney.

Control 3. A guinea pig that had received, three weeks before, an extract prepared from normal rabbit kidney and 1 c.c. of horse serum.

⁵Products of the Pennsylvania Live Stock Sanitary Board, procured through Dr. John Reichel.

⁶Procured from Dr. John Reichel of H. K. Mulford and Co.

Extracts of the kidneys of rabbits which had received respectively twenty-five cubic centimeters of a 2 per cent. solution of gliadin in 0.1 per cent. sodium hydrate, the protein of six billion pneumococci, ten cubic centimeters of tuberculin, and five cubic centimeters each of mallein and mallease, all failed to sensitize guinea pigs to the respective proteins.

The disappointing results of these experiments necessitated a return to the use of egg-albumin and horse serum as the most satisfactory agents to be used in settling the fundamental points of the investigation. The most important of these points was to determine the distribution of the egg-white in the body of the injected rabbit. Does a foreign protein tend to accumulate to a greater extent in the kidney than in other organs, or is it present in the kidney in the same concentration as in other organs; in other words, is the content of foreign protein of an organ dependent merely upon the amount of the circulating blood present in that organ? This point was determined by the experiments summarized in series III and IV, representing the injection of egg-albumin and horse serum respectively.

SERIES III.

Comparison of Sensitizing Power of Blood, Liver, and Kidney after the Intravenous Injection of Egg-White.

Rabbits received 10 c.c. of egg-white diluted with 30 c.c. of salt solution, and were killed after twenty-four, forty-eight, and seventy-two hours, respectively. Blood and extracts of liver and kidneys were injected into guinea pigs. After three weeks each guinea pig received intravenously 4 c.c. of a 1:3 egg-white dilution or intraperitoneally 5 c.c. of a 1:1 dilution.

Guinea pigs.	Time.	Blood.	Liver.	Kidney.	Mode of injection of intoxicating dose.
Group 1	24 hrs.	0	+	++	Intraperitoneal.
Group 2	24 hrs.	++	+++	+	Intravenous.
Group 3	48 hrs.	+++	+	0	Intraperitoneal.
Group 4	48 hrs.	+	++	0	Intravenous.
Group 5	72 hrs.	0	0	0	Intraperitoneal.
Group 6	72 hrs.	0	0	0	Intravenous.

¹ Products of the Pennsylvania Live Stock Sanitary Board, procured through Dr. John Reichel.

² Procured from Dr. John Reichel of H. K. Mulford and Co.

³ Prepared by Dr. J. A. Kolmer according to the method of E. C. Rosenow, *Pneumococcus Anaphylaxis and Immunity*, *Jour. Infect. Dis.*, 1911, ix, 190.

⁴ Product prepared by Drs. T. B. Osborne and H. G. Wells, and obtained through the latter.

SERIES IV.

The same as series III except that horse serum (10 c.c.) was used instead of egg-white.

Guinea pigs.	Time.	Blood.	Liver.	Kidney.	Mode of injection of intoxicating dose. ¹¹
Group 1	24 hrs.	++++	+	++	Intravenous.
Group 2	48 hrs.	++	+	+	Intravenous.
Group 3	72 hrs.	+	0	++++	Intravenous.

These experiments give somewhat irregular results, but in general they indicate that sensitization is dependent on the egg-albumin content of the blood rather than on a retention of egg-albumin in the tissues of the organs. The irregularities in both series are difficult to explain, but such irregularities are not infrequent in sensitization experiments.

To settle the question of whether the egg-white was merely present in the blood or whether it was fixed by the cells of the organ, the experiments summarized in series V were undertaken. In these the sensitizing power of the blood of a rabbit was compared with the same power of extracts of the washed and unwashed kidneys.

The method was as follows. Under the ether anesthesia a rabbit was bled from the carotid artery to secure five cubic centimeters of defibrinated blood. The abdomen was then opened, and the vessels of one kidney were ligated, and the other kidney was washed out with about 1,000 cubic centimeters of physiological salt solution through a cannula in the aorta. Guinea pigs were sensitized with the blood from the carotid and with extracts of the two kidneys.

SERIES V.

Egg-White. Comparison of Sensitizing Power of Blood and of Extracts of Washed and of Unwashed Kidney.

Guinea pigs.	Time.	Blood.	Unwashed kidney.	Washed kidney.	Mode of injection.
Group 1	24 hrs.	++++	+++	+	Intravenous.
Group 2	48 hrs.	+++	+++	+	Intravenous.
Group 3	72 hrs.	++	++	0	Intravenous.
Group 4	96 hrs.	0	++	0	Intravenous.

¹¹ In this and in most of the experiments that follow, the intraperitoneal administration of the intoxicating dose was abandoned on account of the more definite results obtained by the intravenous method.

These results are conclusive. In the normal animal the power of sensitization depends on the egg-albumin content of the blood and not upon a peculiar fixation of the foreign protein in the kidney. It does not necessarily follow that the same is true for all protein substances, but these observations tend, nevertheless, to support the view that injury to the kidney by a particular substance is coincident with the period of elimination of that substance by the kidney, and not to a fixation in the parenchymatous cells.

Another problem, however, remained. Is a foreign protein eliminated as readily by the diseased kidney as it is by the normal kidney? This problem could perhaps have been more exactly solved if the experiments had been made on animals with a chronic renal lesion, but the difficulty of producing the latter with any degree of constancy compelled the use of animals with acute lesions due either to uranium nitrate or to potassium chromate.

The results obtained with three different groups of rabbits suffering from nephritis due to uranium nitrate are combined in the table that follows.

SERIES VI.

The Sensitizing Power of Kidney Extracts from Animals that Received Egg-Albumin After Developing a Uranium Nephritis.

Each rabbit received 0.0075 or 0.015 gm. of uranium nitrate subcutaneously. Twenty-four hours later, when coagulable protein was demonstrable in the urine, each received in the ear vein 10 c.c. of egg-albumin diluted with 30 c.c. of salt solution. The other procedures were the same as in the earlier experiments.

No. of animal.	Time.	Mode of injection.	Result.
Guinea pig 1	24 hrs.	Intraperitoneal.	+++
Guinea pig 2	24 hrs.	Intravenous.	++++
Guinea pig 3	48 hrs.	Intravenous.	o
Guinea pig 4	48 hrs.	Intraperitoneal.	o
Guinea pig 5	48 hrs.	Intravenous.	o
Guinea pig 6	48 hrs.	Intraperitoneal.	o
Guinea pig 7	48 hrs.	Intravenous.	++++
Guinea pig 8	72 hrs.	Intraperitoneal.	++
Guinea pig 9	72 hrs.	Intravenous.	++++
Guinea pig 10	72 hrs.	Intravenous.	++++
Guinea pig 11	72 hrs.	Intravenous.	++++
Guinea pig 12	72 hrs.	Intraperitoneal.	+
Guinea pig 13	72 hrs.	Intravenous.	++
Guinea pig 14	96 hrs.	Intraperitoneal.	o
Guinea pig 15	96 hrs.	Intravenous.	o
Guinea pig 16	96 hrs.	Intravenous.	o

These results are in general similar to those obtained in normal animals, but there is here a greater tendency to sensitization by extracts made as late as the third day (compare series I, III, and V). An acute nephritis due to uranium nitrate apparently lengthens slightly the period during which the kidney (or blood?) may retain a foreign protein. The difference is, however, slight. No explanation is at hand for the curious negative results in four of the five experiments with forty-eight hour extracts.

In another experiment, potassium chromate was used as the renal irritant, and the guinea pigs were sensitized as in series V with blood and with the extracts of washed and unwashed kidneys.

SERIES VII.

Comparison of Sensitizing Power of Blood and of Extracts of Washed and Unwashed Kidneys from Animals that Received Egg-Albumin After Developing a Chromate Nephritis.

Each rabbit was injected subcutaneously with 0.3 gm. of potassium chromate, and on the following day, after coagulable protein had been demonstrated in the urine, received in the ear vein 10 c.c. of egg-white diluted with 30 c.c. of salt solution. Blood was drawn and the extracts of washed and unwashed kidney were made after 1, 2, 3, 4, and 5 days. All intoxicating doses were given intravenously.

Guinea pigs.	Time.	Blood.	Unwashed kidney.	Washed kidney.
Group 1	24 hrs.	++++	++++	++++
Group 2	48 hrs.	+++	++++	++
Group 3	72 hrs.	++	++	+
Group 4	96 hrs.	++++	+++	+
Group 5	108 hrs.	0	++++	0

Here we have definite evidence of a persistence of the egg-albumin in the blood, as is shown by the sensitizing power of the blood and of the extracts of unwashed kidney on the fourth day, a period greater by twenty-four to forty-eight hours (series I, III, and V) than that in the normal animal. The discordant result with the unwashed kidney extract of the fifth day cannot be explained, but it is in keeping with the irregular results seen in other series (series III, IV, and VI). These irregularities are due in all probability to the variations in the susceptibility of the guinea pig to sensitization, but may possibly be due, also, to variations in the power of the rabbits to eliminate a foreign protein.

This variation in the results is the one weak point of this investigation. However, the results, in general, agree, and a comparison of normal animals with those having nephritis shows that in nephritis there is a delay in elimination of about twenty-four to forty-eight hours, and this delay is too constant to be due to experimental errors in the method employed.

DISCUSSION.

This investigation is based on the assumptions that parenterally introduced protein is removed in part by the kidney unaltered, is in part destroyed (digested?) within the tissues, and that the power of the blood or of an extract of the kidney to sensitize against a certain protein is evidence of the presence of that protein in the blood or kidney used for sensitization. With this goes the assumption that until the protein is completely digested or removed, it is being eliminated by the kidney, and that therefore the period of its persistence in the kidney can be determined by sensitization tests. That the egg-albumin is actually eliminated by the kidney and occurs in the urine in a form and in sufficient amount to sensitize guinea pigs is shown by the following experiment.

Ten c.c. of egg-albumin diluted with 0.85 per cent. salt solution were injected into the ear vein of a rabbit and six hours later the urine of the animal was obtained by catheterization. Each of three guinea pigs received 2 c.c. of this urine intraperitoneally. After twenty-four hours the rabbit was again catheterized and the same amount of urine was injected into each of a group of three guinea pigs. Nine days later one guinea pig of each group received in the jugular vein 1 c.c. of egg-albumin diluted with salt solution. The disturbance which resulted was of slight severity and of doubtful significance. At the end of three weeks, however, a similar treatment of the four remaining guinea pigs resulted in typical symptoms and the death of all the animals. On autopsy they showed the characteristic lungs of anaphylaxis. There can be no doubt, therefore, that in the rabbit the intravenous introduction of egg-albumin is followed by its elimination by the kidney in a form capable of causing sensitization. No attempt has been made to determine how long the sensitizing power of the urine persists.

From other experiments, however, there is considerable evidence that the larger part of the injected egg-albumin is eliminated with the urine during the first forty-eight hours after injection. Thus, in the urine of the first day, and to some extent in that of the

second day, there appears on heating either a solid clot or a heavy, coarse, flocculent precipitate which settles rapidly and is quite different from the fine, granular, slowly settling precipitate of the serum proteins of nephritis. After forty-eight hours little or no precipitate is obtained. The possibility that the coagulable protein is in part composed of native serum albumin cannot be denied, but the prompt disappearance of coagulable protein after forty-eight hours is not characteristic of any known acute experimental nephritis giving so large a precipitate in the first twenty-four hours. As normal urine has the power through its non-coagulable protein content, according to Wells and others, to sensitize guinea pigs to serum proteins, the anaphylaxis reaction for such proteins cannot be used as a differential control and has, therefore, not been attempted in this investigation.

It has been assumed, therefore, that most of the coagulable nitrogen of the urine after intravenous injection of egg-albumin is indeed egg-albumin, and in order to determine the rate of its elimination, for comparison with the sensitization tests, determinations of the total nitrogen of the egg-white mixture injected, and of the coagulable protein of the urine, have been made. The following experiment is illustrative.

On April 26, 1912, a rabbit received in the ear vein 40 c.c. of a 1:3 mixture of egg-albumin. The nitrogen of 40 c.c. of this mixture, as estimated by the Kjeldahl method, was 0.191 gm.

Nitrogen in coagulable protein of urine of the first period of 20 hrs... 0.096 gm.

Nitrogen in coagulable protein of urine of the second period of 28 hrs. 0.0441 gm.

Nitrogen in coagulable protein of urine of the third period of 24 hrs.. 0.0054 gm.

Nitrogen in coagulable protein of urine of the fourth period of 24 hrs. 0.005 gm.

Other observations follow:

I. Total nitrogen in 40 c.c. of egg-white dilution (10 c.c. egg-white). 0.178 gm.

Nitrogen in coagulable protein of urine of the first 48 hrs..... 0.094 gm.

Nitrogen in coagulable protein of urine of the second 48 hrs..... 0.017 gm.

II. Total nitrogen in 40 c.c. of egg-white dilution..... 0.177 gm.

Nitrogen in coagulable protein of urine of the first 48 hrs..... 0.106 gm.

Nitrogen in coagulable protein of urine of the second 48 hrs..... 0.004 gm.

It is impossible to say that the coagulable protein of the urine does not contain serum protein, but its prompt decrease after forty-eight hours favors the supposition that it is mainly egg-albumin.

If this interpretation is correct, considerably more than half is eliminated within forty-eight hours and only very small amounts during the third and fourth days; the remainder is, presumably, digested or otherwise changed in the animal tissues. Thus the evidence concerning the elimination of egg-white obtained in this way supports the results of the sensitization experiments, for the stronger anaphylaxis was always obtained with the first and second day extracts, and the weaker reactions and the failures with extracts representing later periods.

In connection with the question of possible sources of error in these experiments another question arises—that of the effect of foreign protein on the normal kidney. If egg-albumin is toxic to the cells of the kidney, the experiments here described as demonstrating elimination in normal animals are really experiments on animals with injured kidneys. Vaughan, it may be remembered, believes that the kidneys of animals receiving egg-white are seriously injured. In the present investigation histological examination of the kidney was made only when an animal died before the regular period set for making extracts. In several instances a definite exudate about the glomerular tuft, markedly distending Bowman's capsule, has been seen. Whether this represents an exudate of serum albumin or of the foreign egg-albumin coagulated by the fixing agent we have found no means of determining. The prompt disappearance (after forty-eight to seventy-two hours) of coagulable protein from the urine has, however, seemed to indicate that the kidney is not seriously injured by a single injection and that the fine granular precipitate in the glomerular spaces is, therefore, in all probability egg-albumin.

SUMMARY.

Extracts of the kidneys of normal rabbits prepared one, two, three, and four days after the intravenous injection of egg-albumin and horse serum have the power to sensitize guinea pigs to a second injection of these proteins. The sensitization by first and second day extracts was constant and intense, that by the third day extracts was less marked and sometimes was not evident, and that by the fourth day extracts was only occasional, and when present was always weak.

Comparative studies of the power of the blood, liver, and kidney to sensitize, indicate that this sensitization depends on the content of foreign protein in the circulating blood and not upon its accumulation or fixation in the tissues of an organ. This opinion is supported by other experiments in which the sensitizing power of the blood and of the extracts of unwashed kidneys was compared with the sensitizing power of extracts of washed kidney.

The weak sensitizing power of washed kidney extract is taken as evidence that foreign proteins of the kinds used are not held in the tissues of the kidney, and if these results may be applied to nephrotoxic proteins, it follows that nephritis is not due to selective and persisting fixation of a protein by the renal cells, but is due to the action of such protein merely during the process of its elimination.

In experimental acute nephritis of the type due to uranium nitrate, the power of sensitization to egg-albumin is prolonged for twenty-four hours, and in the chromate type for forty-eight hours, thus indicating that in nephritis, of the acute type at least, the elimination of a foreign protein is delayed.

Attempts to study by the same methods the elimination of vegetable and bacterial proteins have failed.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

I. REACTIONS TO HEMOLYTIC SERUM AT VARIOUS INTERVALS AFTER SPLENECTOMY.*

By RICHARD M. PEARCE, M.D., J. H. AUSTIN, M.D., AND
E. B. KRUMBHAAR, M.D.

(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.)

This investigation was originally undertaken for the purpose of determining the relation which might exist between the tumefaction of the spleen and the jaundice so characteristic of acute blood destruction by hemolytic poisons. As the work progressed, however, it became evident that a problem of much wider scope was before us and that it was necessary to study the general relation of the spleen to blood destruction and its sequelæ.

In the early experiments in which was studied the effect of hemolytic serum in the absence of the spleen, very definite and constant results in connection with the question of jaundice were obtained, but, as the time after splenectomy increased, other factors entered to such a degree that it became necessary to attack the problem from many sides. In order, therefore, to prepare the way for a rational consideration of the many aspects of the problem, this communication will be devoted to a general statement (1) of the relation between the hemoglobinuria and the jaundice occurring in normal animals and in splenectomized animals receiving hemolytic serum, and (2) of certain changes in the blood and urine apparently dependent on splenectomy. The explanation of these will be discussed in subsequent papers.

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METHODS.

The blood-destroying agent employed in the present series of experiments was hemolytic immune serum prepared by injecting rabbits with defibrinated blood of the dog. Five injections of five to ten cubic centimeters at intervals of five to seven days gave, as a rule, a very active hemolytic serum.

The operation of splenectomy was done aseptically under ether anesthesia. Female dogs were used almost exclusively in order that the tests for hemoglobin and bile might be made on urine obtained by catheterization. This we have found to be a most important precaution, for not infrequently the cage urine of apparently healthy dogs contains bile, and admixture of biliary pigments cannot be avoided if diarrhea or vomiting is present. All injections of hemolytic serum were made under ether anesthesia into a small superficial vein of the leg or into the jugular vein. Each experiment on a splenectomized animal was controlled by the injection of a normal animal with the same serum. When the animals were of approximately the same weight they received the same amounts of serum; but when the weight varied more than half a kilo, they received, with a few exceptions, corresponding doses per kilo of body weight.

The urine of all animals was examined for coagulable protein, hemoglobin, and bile pigments. In regard to the latter it may be said that little attention has been paid to the staining of the sclera or to the general staining of the tissue as evidence of jaundice. The appearance of bile pigments in the urine has been taken as the surest indication of jaundice, and the simple tests (Gmelin, Rosenbach) have, as a rule, been used, though in doubtful cases the more delicate tests were tried, and in several series tests for bile salts were made as a matter of routine.

**THE FAILURE OF JAUNDICE DURING SHORT PERIODS AFTER
SPLENECTOMY.**

The effect of a powerful hemolytic serum upon the recently splenectomized animal is not widely different from that upon the normal animal. In both the blood is laked freely and death results in a few hours, thus indicating no increase in resistance in the splenectomized animal. This is shown in experiment I.

EXPERIMENT I.

A normal dog weighing 8,220 gm., and a dog splenectomized three days before, weighing 9,025 gm., received hemolytic serum intravenously in proportions of 0.5 c.c. per kilo of body weight. Both vomited shortly after receiving the serum, became progressively weaker, and died after a few hours. No urine could be obtained by catheterization and none was found in the bladder of either after death.

At autopsy both animals presented certain lesions in common, e. g., fluid in the peritoneal cavity, congestion of the liver, and edema of the lymph nodes, kidneys, and gall-bladder. The control had the greatly distended, soft, black, spodogenous spleen that is characteristic of acute blood destruction. In the splenectomized animal there were petechial hemorrhages of the serous membranes and of the mucosa of the intestine; these were not seen in the control dog. The heart and blood-vessels contained loose clots, and the serum of each animal showed, after centrifugalization, deep staining with hemoglobin.

Histological examination of tissues from the control dog showed intense congestion of the liver with general necrosis and hyaline thrombi in the blood-vessels. The sinuses of the spleen were distended by fused hyaline masses of red blood cells and the pulp was more or less necrotic. The vessels of the kidney contained hyaline masses, and the epithelium of the tubules showed granular degeneration. The lymph nodes were edematous and in them were small areas of necrosis. The liver, kidney, and lymph nodes of the splenectomized dog presented a similar histological picture.

The extreme opposite of the above experiment is shown in experiment II, in which a weak serum was used.

EXPERIMENT II.

Effect of Hemolytic Serum Administered Three Days after Splenectomy, with Control.

Date.	Dog 11. Weight 10,000 gm.	Dog 13. Weight 8,990 gm.
Dec. 11, 1911.	Splenectomy.	Control.
Dec. 14, 1911.	Urine normal; 2.5 c.c. hemolytic serum in vein.	Urine normal; 2.5 c.c. hemolytic serum in vein.
Dec. 15, 1911.	Urine normal.	Bile test positive; no hemoglobin, no albumin.
Dec. 16, 1911.	Urine normal; 4.5 c.c. of same serum in vein.	Bile test positive; no hemoglobin, no albumin; 4.5 c.c. of same serum in vein.
Dec. 17, 1911.	Trace of albumin; no bile, no hemoglobin.	Bile test positive; albumin present, no hemoglobin.
Dec. 18, 1911.	Trace of albumin; no bile, no hemoglobin.	Bile test positive; albumin present, no hemoglobin.
Dec. 19, 1911.	Trace of albumin; no bile, no hemoglobin.	Bile test positive; albumin present, no hemoglobin.
	Ligation of common bile duct under ether anesthesia.	Died, under ether, during operation to remove spleen.
Dec. 20, 1911	Bile pigments in urine.	
Dec. 21-22, 1911.		
Dec. 23, 1911.	Bile pigments in urine increasing. Bile pigments in urine increasing. Killed by chloroform.	

In this experiment the hemolytic serum was not powerful enough to cause a destruction of blood of sufficient grade to produce hemoglobinuria, although it did cause in the control animal enough destruction to produce jaundice; on the other hand, the splenectomized animal was free from jaundice.

Three possible explanations of this difference in results are at hand: first, that the absence of the spleen has an influence on the elimination of bile; second, that splenectomy influences in some way the series of changes by which blood pigments are converted into bile pigments; and third, that owing to the difference in the weight of the animals, the splenectomized animal received a relatively smaller dose, too small, perhaps, to produce a degree of blood destruction capable of causing bile pigments to appear in the urine.

The first of these possible explanations may be dismissed at once in view of the appearance of bile pigments in the urine of the splenectomized dog within twenty-four hours after ligation of the common duct. The second is the main problem discussed in this and succeeding papers. The third explanation may be dismissed in view of the result in experiment III in which a stronger serum was used and the amount injected was adjusted to the weight of the animals employed.

EXPERIMENT III.

Effect of Hemolytic Serum Six Days after Splenectomy, with Control.

Date.	Dog 3. Weight 6,400 gm.	Dog 7. Weight 10,580 gm.
Dec. 14, 1911.	Splenectomy.	Control.
Dec. 20, 1911.	Urine normal.	Urine normal.
	0.5 c.c. serum per kilo.	0.5 c.c. serum per kilo.
Dec. 21, 1911.	Hemoglobinuria; no bile pigment.	Hemoglobinuria; marked jaundice.
Dec. 22, 1911.	Hemoglobinuria; no bile pigment.	Hemoglobinuria; marked jaundice.
Dec. 23, 1911.	Hemoglobinuria; no bile pigment.	No hemoglobinuria; marked jaundice.
Dec. 24, 1911.	Trace of hemoglobinuria; no bile pigment.	No hemoglobinuria; marked jaundice.
Dec. 25, 1911.	No hemoglobinuria; no bile pigment.	No hemoglobinuria; marked jaundice.
Dec. 26-27, 1911.	Faint trace of bile pigment.	Much bile pigment in urine.
Dec. 28, 1911.	No bile pigment.	Much bile pigment in urine.
Dec. 29, 1911.	No bile pigment.	Much bile pigment in urine.

At autopsy, dog 3 showed no bile staining of the tissues. All the organs appeared normal and the lymph nodes were not enlarged. The gall-bladder was

distended by a large amount of dark green bile. Dog 7 presented the usual large, swollen, black, spodogenous spleen; the kidneys, on section, were brownish in color, the lymph nodes were swollen, and the gall-bladder was filled with dark brown bile. Bile staining of the tissue could not be demonstrated.

In this experiment the injection of serum caused well marked hemoglobinuria in both dogs, accompanied in the normal dog by a persisting excretion of large amounts of bile pigments in the urine, but without evidence of jaundice in the splenectomized dog. On only two days did the splenectomized dog show the faintest trace of bile in the urine, and on these days the reaction was so faint as to be classed as doubtful.

EXPERIMENT IV.

Effect of Serum Fifteen Days after Splenectomy.

Date.	Dog 4. Weight 9,200 gm.	Dog 6. Weight 8,800 gm.
Nov. 14, 1911.	Splenectomy.	Control.
Nov. 25-28, 1911.	Urine normal.	Urine normal.
Nov. 29, 1911.	0.5 c.c. serum per kilo.	0.5 c.c. serum per kilo.
Nov. 30, 1911.	Marked hemoglobinuria; no bile pigment.	Marked hemoglobinuria; bile pigment abundant.
Dec. 1, 1911.	No hemoglobinuria; no bile pigment.	Trace of hemoglobinuria; much bile pigment.
Dec. 2, 1911.	No hemoglobinuria; no bile pigment.	No hemoglobinuria; much bile pigment.
	Died.	Chloroformed.

At autopsy both dogs showed focal necroses of the liver, brownish discoloration of the kidneys, and swollen edematous lymph nodes. The spleen of the control dog presented the usual spodogenous tumefaction. In this animal the lighter tissues were stained a faint yellow, which was not the case in the splenectomized animal. In each, the gall-bladder was distended and contained thick, blackish brown bile.

These experiments have one uniform result; in the splenectomized animal, jaundice failed to follow hemoglobinuria. Moreover, in the splenectomized dog, jaundice failed to follow those grades of blood destruction which, as in experiment II, caused jaundice in the normal animal, but were too slight to cause hemoglobinuria.

The literature of jaundice contains, so far as we are aware, only one series of observations on the effect of hemolytic serum in splenectomized animals, and from these observations erroneous con-

clusions have been drawn. Joannovics,¹ in his studies of the hemolytic jaundice caused by toluylendiamin, found, as had Banti² and Vast,³ that splenectomized dogs require larger doses of the drug to cause jaundice than do normal animals and he agreed, therefore, with the conclusion of Vast, that for the destruction of the blood cells by toluylendiamin, the intervention of the spleen is necessary. In the course of his work, the effect of hemolytic immune serum was tried on three splenectomized dogs. The spleen of two had been removed eight days before the injection of the serum; the time of removal in the third is not stated. Two of the animals died within a few hours and one was killed on the ninth day. In none did hemoglobinuria or jaundice appear, and Joannovics concludes that the presence of the spleen is essential for the production of the hemoglobinuria due to hemolytic serum.

That this is not true is shown by our experiments (III and IV) in which hemoglobinuria was well marked. The different result in Joannovics' experiments was probably due to the method of injection, the subcutaneous, which does not yield the same degree of blood destruction as does the intravenous injection. It is of interest, however, in view of our results, that in his experiment (VII) in which a dog received, eight days after splenectomy, a relatively large dose of serum (nearly one cubic centimeter per kilo), no jaundice occurred during a period of nine days.

Experiments having for their object an explanation of the failure of hemolytic serum to cause jaundice during short periods after splenectomy are presented elsewhere. It may be stated here, however, that the phenomenon is not due to an influence of the spleen upon the transformation of hemoglobin,⁴ but is apparently dependent on the changes in the blood consequent upon splenectomy.⁵

¹ Joannovics, G., Experimentelle Untersuchungen über Ikterus, *Ztschr. f. Heilk.*, 1904, xxv, 25.

² Banti, cited by Joannovics, *loc. cit.*

³ Vast, cited by Joannovics, *loc. cit.*

⁴ Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., II. The Relation of Hemoglobinemia to Hemoglobinuria and Jaundice in Normal and Splenectomized Animals, *Jour. Exper. Med.*, 1912, xvi, 375.

⁵ Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, in press.

Moreover, it may be added that if splenectomy is not accompanied by anemia, on the administration of hemolytic serum jaundice occurs in the splenectomized as in the normal animal.

THE INCREASED RESISTANCE OF RED CELLS TO HEMOLYSIS AFTER SPLENECTOMY.

As the time after splenectomy lengthened, a new factor came into consideration. This was the increased resistance of the red blood cells to the action of a hemolytic serum, observed first after twenty-seven days.

EXPERIMENT V.

Increased Resistance to Hemolysis Twenty-seven Days after Splenectomy.

Date.	Dog 1. Weight 7,940 gm.	Dog 8. Weight 6,880 gm.
Nov. 10, 1911.	Splenectomy.	Control.
Dec. 5, 1911.	Urine normal.	Urine normal.
Dec. 6, 1911.	Urine normal.	Urine normal.
Dec. 7, 1911.	0.5 c.c. serum per kilo into vein. No hemoglobin or bile pigment in urine; large amount of coagulable protein. A second injection of 5 c.c. serum into vein.	0.5 c.c. serum per kilo into vein. Severe hemoglobinuria; much bile pigment in urine. No second injection.
Dec. 8, 1911.	Well marked hemoglobinuria; faint trace of bile pigments.	Slight hemoglobinuria; moderate bile pigment.
Dec. 9, 1911.	No hemoglobinuria; trace of bile. Chloroformed.	No hemoglobinuria; trace of bile. Chloroformed.

At autopsy dog 1 showed numerous focal necroses in the liver, congested and hemorrhagic lymph nodes, and brownish discoloration of the kidneys. The gall-bladder contained a very dark bile.

Dog 8 had the typical, firm, swollen, bluish red, spodogenous spleen and also focal necroses of the liver, congested edematous lymph nodes, and brownish discoloration of the kidneys. The gall-bladder contained a black bile thicker than that of dog 1.

In this experiment the increased resistance of the splenectomized dog's corpuscles was very clearly shown. Not only did this animal receive the same relative dose per weight, but actually 0.5 of a cubic centimeter more than did the control animal. Moreover, a second injection of five cubic centimeters caused, in so far as hemoglobinuria may be taken as an index, a less severe grade of blood destruction than in the control animal. In contrast to the previous

experiments it will be noted that the urine of the splenectomized animal contained bile pigments, but not as great an amount as the urine of the control animal.

Another experiment of the same character follows.

EXPERIMENT VI.

Increased Resistance to Hemolysis Sixty-five Days after Splenectomy.

Date.	Dog 10. Weight 9,720 gm.	Dog 22. Weight 6,710 gm.
Dec. 9, 1911.	Splenectomy.	Control.
Feb. 12, 1912.	Urine normal.	Urine normal.
Feb. 13, 1912.	0.25 c.c. serum per kilo into vein.	0.25 c.c. serum per kilo into vein.
Feb. 14, 1912.	Urine free from hemoglobin and bile pigment.	No hemoglobinuria; bile pigments present.
	1 c.c. same serum per kilo into vein.	1 c.c. per kilo of same serum into vein.
Feb. 15, 1912.	No hemoglobinuria; no bile pigment.	Marked hemoglobinuria; much bile pigment.
Feb. 16, 1912.	No hemoglobinuria; no bile pigment.	No hemoglobinuria; much bile pigment.
	2 c.c. per kilo of another serum into vein.	No third injection. Spleen excised.
Feb. 17, 1912.	Hemoglobinuria; faint trace of bile pigment.	Well marked bile reaction.
Feb. 18, 1912.	No hemoglobinuria; faint trace of bile (?).	Well marked bile reaction.
Feb. 19, 1912.	No hemoglobinuria; faint trace of bile (?).	Well marked bile reaction.
	Common bile duct ligated.	
Feb. 20-21, 1912.	Large amount of bile pigment in the urine.	Well marked bile reaction.
	Chloroformed.	Chloroformed.

At autopsy dog 10 showed general jaundice and fatty changes in the liver, kidneys, and heart. In dog 22, fatty changes were evident, but were not so marked, and no definite bile staining of the tissues could be seen.

This experiment demonstrates most conclusively the increased resistance of the red blood cells of a splenectomized animal to hemolysis. A small dose (0.25 of a cubic centimeter per kilo) of a weak serum caused in the sound dog sufficient destruction of red cells to lead to the appearance of bile pigments in the urine; the splenectomized animal was not affected. A larger dose (one cubic centimeter per kilo) of the same serum produced hemoglobinuria in the control, but no change in the splenectomized animal. Finally, a third injection of two cubic centimeters of serum per kilo, an unusually large dose, was necessary before hemoglobinuria was produced in the splenectomized animal.

This evidence of increased resistance in the splenectomized animal was supported by tests *in vitro*, both as regards spontaneous hemolysis in salt solutions of different strengths, and also as regards the hemolytic power of the serum used for injection.

Incidentally, these two animals were used to demonstrate (1) that obstructive jaundice can readily be produced in the splenectomized dog (see also experiment I) by ligating the common duct, and (2) that an existing hemolytic jaundice does not immediately lessen after removal of the spleen.

The determination of the cause of this increased resistance of the red corpuscles after splenectomy is necessarily an important part of our problem. That the splenectomized animal is more resistant than the normal animal to the action of hemolytic poisons, that is, that the splenectomized dog will bear larger doses, has been noted by Banti, Vast, and Joannovics in their work with toluylendiamin, and was also observed by Pugliese and Luzzatti,⁶ in their study of the action of pyrocin.

Actual demonstration by exact methods of an increased resistance of red cells after splenectomy has been shown, so far as we are aware, only by Chalier and Charlet,⁷ though these investigators quote also Bottazi⁸ and Viola.⁹ The differences noted by Chalier and Charlet as the result of testing the fragility of the cells of oxalated blood in different strengths of salt solution are slight, but this may be due to the fact that none of their tests appear to have been made later than ten to twelve days after splenectomy. They found in three dogs that complete hemolysis occurred in solutions varying from 0.32 to 0.36 as compared to 0.32 to 0.4 in five normal dogs.

In our work we have used fresh blood obtained by puncture and have allowed it to fall into tubes as it flowed from the wound, one drop in each tube. These tubes contained salt solution decreasing

⁶ Pugliese, A., and Luzzatti, T., Contributions à la physiologie de la rate, *Arch. ital. de biol.*, 1900, xxxiii, 349.

⁷ Chalier, J., and Charlet, L., État de la résistance globulaire chez l'animal normal et splénectomisé, *Jour. de physiol. et de path. gén.*, 1911, xiii, 728.

⁸ Bottazi, cited by Chalier, J., and Charlet, L. (with reference to Morat, J.-P., and Doyon, M., *Traité de physiologie*, Paris, 1904, i, 605), *loc. cit.*

⁹ Viola, cited by Chalier, J., and Charlet, L. (with reference to Morat, J.-P., and Doyon, M., *Traité de physiologie*, Paris, 1904, i, 605), *loc. cit.*

by twentieths of 1 per cent. from 0.7 to 0.2 per cent. In normal dogs we have found that hemolysis is complete in 0.35 or 0.4 per cent. solution, with partial hemolysis, as a rule, in the next higher tube. Only once in our experiments with the blood of normal dogs has complete hemolysis occurred in 0.3 per cent. solution. In splenectomized animals, on the other hand, if a period of at least nine days has elapsed, complete hemolysis occurs in 0.25 or 0.3 per cent. solution with partial hemolysis in the next higher tube, but the hemolysis is never complete in solutions above 0.3 per cent. In a few instances in which this test has been applied both before and after splenectomy, the increase in resistance corresponded always to a difference of 0.1 per cent. in the salt solution used. No increase in resistance was noted before the ninth day, and after this period the resistance did not increase with lapse of time up to seven and a half months, which in our series is the longest period after splenectomy.

Detailed observations having for their object the determination of the cause of this increased resistance and its relation to blood destruction and regeneration will be presented in later communications.¹⁰

THE OCCURRENCE OF SPONTANEOUS JAUNDICE SEVERAL MONTHS AFTER SPLENECTOMY.

In view of the difficulty of causing hemolytic jaundice in the early periods, the occurrence of spontaneous jaundice several months after splenectomy was entirely unexpected. Well marked coloring of the urine by bile pigment has been seen in two animals. In one it was noted 103 days after splenectomy and in the other after seven and a half months. How soon after the removal of the spleen this spontaneous pigmentation may occur we cannot say, for in the early part of the investigation many of the dogs were watched for only a few days after operation, and were then set aside without routine examination of the urine. Thus, the occurrence of bile in the urine was, in several of the long period splenectomies, not ob-

¹⁰ Karsner, H. T., and Pearce, R. M., IV. A Study, by the Methods of Immunology, of the Increased Resistance of the Red Blood Corpuscles after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, in press.

served until the period of study preliminary to the injection of serum. For this reason we did not determine when the bile pigments first appeared in the urine of the animals mentioned above. Since our attention was called to this phenomenon, however, all animals have been carefully watched, but large amounts of bile pigments have not been observed in the urine of animals splenectomized for periods of less than three months. In two instances, however, faint traces were found after thirty days. On the other hand, we have had only four long period splenectomies to study. In two of these (three and one half and seven and one half months) spontaneous jaundice occurred and in two (nine and one half and ten months) it was not observable.

The supposition that this spontaneous jaundice might be due to a diminished resistance and to a spontaneous hemolysis of red cells is contra-indicated by the fact that the red cells of both animals presenting jaundice showed an increased resistance to lysis by both salt solution and hemolytic serum. The following experiment will illustrate this.

EXPERIMENT VII.

Spontaneous Jaundice One Hundred and Three Days after Splenectomy, with Increased Resistance of the Red Cells.

Date.	Dog 2. Weight 10,290 gm.	Dog 29. Weight 7,250 gm.
Nov. 14, 1911.	Urine normal.	Control.
Nov. 15, 1911.	Splenectomy.	Urine normal.
Feb. 25, 1912.	Urine deeply pigmented by bile.	0.25 c.c. serum per kilo into vein.
Feb. 26, 1912.	0.25 c.c. serum per kilo into vein.	Marked hemoglobinuria; fairly well marked bile reaction.
Feb. 27, 1912.	No hemoglobinuria; much bile pigment.	Slight hemoglobinuria; much bile pigment.
Feb. 28, 1912.	No hemoglobinuria; much bile pigment.	Urine lost.
Feb. 29, 1912.	No hemoglobinuria; much bile pigment.	Much bile pigment; no hemoglobinuria.
Mar. 1, 1912.	No hemoglobinuria; much bile pigment.	
Experiment discontinued.		

At autopsy the splenectomized animal presented no disturbance of the liver or biliary passages capable of explaining the jaundice as being due to obstruction.

In a second animal a persisting and abundant pigmentation of the urine was noticed seven and a half months after splenectomy. The increased resistance of the red cells was shown by hemolysis in 0.3

per cent. salt solution, whereas the cells of the control animal were hemolyzed in 0.4 per cent. Hemoglobinuria was brought about in the control dog by an injection of 0.5 of a cubic centimeter of hemolytic serum per kilo, while in the splenectomized animal hemoglobinuria did not occur until the dose was raised to one cubic centimeter per kilo.

That the jaundice in this particular animal was not associated with an anemia is shown by the fact that the blood examination gave a red blood cell count of 6,120,000 and a hemoglobin content of 97 per cent. Our attempts to explain this phenomenon are presented in the third paper¹¹ of this series.

SUMMARY.

This investigation brings out three points.

1. During the first three or four weeks after splenectomy hemoglobinuria due to hemolytic serum was not followed by jaundice.
2. One month and more after splenectomy the red cells had an increased resistance to hemolysis.
3. Three and a half months after splenectomy and even later, jaundice occasionally occurred spontaneously.

Attempts to explain these three phenomena have been based on the following possibilities: (1) that some function of the spleen is essential for the degradation of hemoglobin or for the elaboration of its derivatives; (2) that there is some interrelation between the spleen and the blood-forming organs or between the spleen and the blood-disintegrating mechanism, or perhaps both interrelations exist; (3) that there is a disturbance of the hemolyzing or bile-forming function of the liver dependent on the blood changes following splenectomy.

This multiplicity of problems and likewise of possible explanations has rendered the presentation of the explanatory work in one communication extremely difficult. In the interests of clearness, therefore, the various phases are dealt with in separate papers.

¹¹ Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., *loc. cit.*

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

II. THE RELATION OF HEMOGLOBINEMIA TO HEMOGLOBINURIA AND JAUNDICE IN NORMAL AND SPLENECTOMIZED ANIMALS.*

BY R. M. PEARCE, M.D., J. H. AUSTIN, M.D., AND
A. B. EISENBREY, M.D.

(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.)

This publication is the second of a series on the general subject of the influence of the spleen on blood degeneration and regeneration and the production of hemolytic jaundice, and has for its object the establishment of a satisfactory theory to explain, on the one hand, the quantitative relations of hemoglobinuria and jaundice, and, on the other, the relative importance of the liver and kidneys in removing free hemoglobin from the circulating blood.

The main investigation, to which the present study bears only an incidental relation, was undertaken for the purpose of determining the relation, if any, which may exist between the peculiar (spodogenous) enlargement of the spleen and the jaundice so characteristic of acute destruction of the blood by hemolytic agents.

In early experiments in which hemolytic serum was administered to dogs shortly after splenectomy, jaundice not infrequently failed to appear. The first, and perhaps natural conclusion was that the spleen is in some way concerned in the preparation of hemoglobin for action by the liver cells, but after repeated attempts had been made to explain the phenomenon it became evident that the problem was not easy of solution and that fundamental observations on the fate of free hemoglobin in the circulating blood were necessary as

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a basis for further investigation. The study of the fate of known quantities of hemoglobin injected into normal and splenectomized animals was therefore undertaken, and it is the results of this study which are here presented. The points studied were: (1) the degree of hemoglobinemia necessary in order to recognize free hemoglobin in the serum; (2) the degree of hemoglobinemia necessary for the escape of hemoglobin through the kidneys; (3) the percentage of hemoglobin eliminated by the kidneys; (4) the degree of retention of hemoglobin necessary to cause jaundice; (5) the influence of the absence of the spleen on the elimination or retention of hemoglobin.

METHODS.

Defibrinated dog blood was hemolyzed with distilled water, sodium chlorid was added to render the solution isotonic with dog blood, the diluted blood was centrifugalized, and the hemoglobin content was determined by Fleischl's method. Definite amounts of the hemoglobin solution, always freshly prepared, were allowed to flow from a burette into a small branch of the femoral vein. The first appearance of hemoglobin in the urine was determined by a catheter in the bladder only or by a second catheter in one ureter. In order to aid the flow of urine each dog received 300 cubic centimeters of water by stomach tube. From time to time the skin was punctured and the blood was drawn into capillary tubes to determine how early free hemoglobin appeared in the serum.

The elimination of hemoglobin in the urine was estimated by rendering the urine acid with hydrochloric acid to about N/10 and comparing this solution of acid hematin, suitably diluted, with a 1 per cent. solution made according to the Sahli method from blood containing 100 per cent. of hemoglobin by the Fleischl scale. A Duboscq colorimeter was used for making the comparison.

Quantities of hemoglobin are designated throughout the paper in grams calculated on the assumption, for the sake of convenience, that blood giving a reading of 100 per cent. by the Fleischl scale contains 14 per cent. of hemoglobin. This figure is, of course, only approximately correct, but as relative quantities are of importance in our work, an approximate determination of the absolute quantities of hemoglobin is sufficient.

It is of the utmost importance that all urine containing hemoglobin be placed promptly in the refrigerator until the colorimetric estimation is made. If such urine is allowed to stand a few hours at room temperature a green precipitate of a hemoglobin derivative is formed which, when treated with sodium chlorid and glacial acetic acid, gives the hemin test.

The experiments were made almost exclusively on female dogs, in order that urine obtained by catheterization might be available for the bile tests. This has been found to be most important on account of the small amounts of bile pigments occurring in the urine under the condition of these experiments. In all experiments ether anesthesia was employed.

The earlier experiments, which are given below, were made with the object of determining approximately (a) how much free hemoglobin is necessary to stain the serum, (b) how much must be introduced to cause its appearance in the urine, and (c) to gain some idea of the time relations.

Experiment I.—Normal dog, female; weight 8,360 gm. Ether anesthesia; cannula in left femoral vein; catheter in bladder; 300 c.c. of water by stomach tube.

Feb. 21, 1912. 3.20 P. M. Urine normal.

3.22 P. M. Blood serum clear.

3.25 P. M. 10 c.c. of hemolyzed blood injected (equal to 0.32 gm. of hemoglobin).

3.30 P. M. Blood serum clear.

3.32 P. M. 10 c.c. of hemolyzed blood injected.

3.37 P. M. Blood serum clear.

3.39 P. M. 10 c.c. of hemolyzed blood injected.

3.40 P. M. Much albumin in urine.

3.44 P. M. Blood serum faintly tinged with hemoglobin.

3.51, 3.58, 4.05, 4.12, 4.19, 4.26 P. M. Blood serum deeply stained with hemoglobin.

3.46, 3.53, 4.00, 4.07, 4.14, and 4.21 P. M. 10 c.c. of hemolyzed blood injected.

3.55, 4.15, 4.21 P. M. Much albumin in urine.

4.22 P. M. Hemoglobin in urine.

5.23 P. M. Hemoglobin in urine decreasing in amount. Total elimination of hemoglobin = 0.18 gm.

8.00 P. M. Urine free of hemoglobin.

Feb. 22-29, 1912. Urine free of hemoglobin; no bile pigment; slight trace of albumin on 22d and 23d; none after this.

Summary.—The total amount of hemoglobin injected was 2.91 gm. (0.35 gm. per kilo); the total elimination by the kidneys was 0.18 gm. The hemoglobin

retained was $2.91 - 0.18 = 2.73$ gm., or 0.33 gm. per kilo. Hemoglobin was demonstrated in the blood serum after a lapse of nineteen minutes when 30 c.c. of the solution (0.97 gm. of hemoglobin) had been injected, and appeared in the urine after fifty-seven minutes when 90 c.c. of the solution (2.91 gm. of hemoglobin) had been introduced. Despite the great retention of hemoglobin, bile pigments did not appear in the urine.

Experiment II.—Normal dog, female; weight 11,090 gm. Ether anesthesia; cannula in branch of right femoral vein; catheter in bladder; 300 c.c. of water by stomach tube.

Feb. 29, 1912. 3.57 P. M. Urine normal.

4.01 P. M. Blood serum clear.

4.02-4.07 P. M. 20 c.c. of hemolyzed blood injected, equal to 0.49 gm. of hemoglobin.

4.07 P. M. Blood serum clear.

4.09-4.15 P. M. 20 c.c. of hemolyzed blood injected.

4.10 P. M. Some albumin in urine.

4.14 P. M. Blood serum clear.

4.16-4.18 P. M. 20 c.c. of hemolyzed blood injected.

4.20 P. M. Much albumin in urine.

4.21 P. M. Blood serum showed a trace of hemoglobin.

4.23-4.26 P. M. 20 c.c. of hemolyzed blood injected.

4.28 P. M. Blood serum deeply colored by hemoglobin.

4.30 P. M. 10 c.c. of hemolyzed blood injected.

4.32 P. M. Large amount of albumin in urine.

4.33 P. M. Hemoglobin present in urine.

4.35 P. M. Blood serum deeply colored by hemoglobin.

March 1, 1912. The cage urine showed a trace of albumin, no bile pigment, and no hemoglobin. Urine by catheter at 10 A. M. showed no albumin.

Summary.—The total amount of hemoglobin injected was 2.19 gm. (0.20 gm. per kilo); the total elimination by the kidney was 0.05 gm. of hemoglobin. The amount retained was, therefore, $2.19 - 0.05 = 2.14$ gm. of hemoglobin, or 0.19 gm. per kilo. Hemoglobinuria was evident after a lapse of nineteen minutes when 60 c.c. of the solution (1.46 gm. of hemoglobin) had been injected, and hemoglobin appeared in the urine after thirty-one minutes when 90 c.c. of solution (2.19 gm. of hemoglobin) had been injected. Although nearly all the hemoglobin was retained, bile pigment did not appear in the urine.

Experiment III.—Normal dog, female; weight 11,040 gm. Ether anesthesia; cannula in branch of femoral vein; catheter in bladder; 300 c.c. of water by stomach tube.

March 1, 1912. 2.30 P. M. Urine normal.

2.34 P. M. Blood serum clear.

2.35-2.38 P. M. 20 c.c. of hemolyzed blood injected (equal to 0.80 gm. of hemoglobin).

2.40 P. M. Blood serum showed faint trace of hemoglobin.

2.42-2.45 P. M. 10 c.c. of hemolyzed blood injected.

2.47 P. M. Blood serum showed faint trace of hemoglobin.

2.49-2.51 P. M. 10 c.c. of hemolyzed blood injected.

2.52 P. M. Hemoglobin present in urine.

2.54 P. M. Serum showed much hemoglobin.

March 2, 1912. 9.00 A. M. Urine obtained by catheter showed no hemoglobin, no bile pigment, and no albumin. Cage urine contained 0.15 gm. of hemoglobin.

12.00 M. Urine obtained by catheter showed no hemoglobin, no bile pigment, and no albumin.

March 3, 1912. Urine normal.

Summary.—Hemoglobin appeared in the serum five minutes after the beginning of the injection of 20 c.c. of the solution (0.80 gm. of hemoglobin), and was present in the urine after seventeen minutes, when 40 c.c. (1.60 gm. of hemoglobin) had been injected. The total amount of hemoglobin introduced was 1.6 gm. (0.14 gm. per kilo). The total amount eliminated was 0.15 gm. The amount retained was, therefore, 1.45 gm. (0.13 gm. per kilo). As in the other experiments, the retained hemoglobin did not appear in the urine as bile pigment.

In order to determine precisely the moment when the kidney begins to excrete hemoglobin, the following experiments, in which a single large injection of hemoglobin was given, were undertaken.

Experiment IV.—Normal dog, female; weight 7,100 gm. Ether anesthesia; cannula in left external jugular vein; catheter in bladder; cannula in left ureter projecting into pelvis of kidney; 300 c.c. of water by stomach tube.

March 2, 1912. 3.29-3.33 P. M. 106 c.c. of hemolyzed blood injected (= 0.34 gm. of hemoglobin).

3.39½ P. M. Hemoglobin appeared simultaneously in both vesical catheter and ureteral cannula.

Experiment V.—In another experiment of the same type a hemoglobin solution containing 0.32 gm. of hemoglobin was injected into the jugular vein between 4.04 and 4.06 P. M. Hemoglobin appeared in the ureteral cannula at 4.14½ P. M., and in the vesical catheter a few seconds later.

In these last experiments, if we take experiment IV for purposes of comparison, it is obvious that the hemoglobin concentration of the blood, determining the threshold of the kidney for hemoglobin, was reached at some time between 3.29 and 3.33 P. M. The detection of hemoglobin in the ureter and bladder was possible at 3.39½ P. M. It is evident from this that hemoglobin can be detected in the bladder about eight minutes after the threshold value of the kidney is reached. We may assume, therefore, that in experiments I, II, and III the threshold of the kidney for hemoglobin was reached eight minutes before hemoglobin appeared in the urine; that is, in experiment I at 4.14 P. M., at which time 2.59 grams of hemoglobin had been injected; in experiment II, at 4.25 P. M., at which time 1.95 grams of hemoglobin had been injected; and in ex-

periment III, at 2.44 P. M., at which time 1.2 grams of hemoglobin had been injected.

The question at once arises as to the reason for the apparent differences in these threshold values, experiments II and III being on the same animal. It is to be found, we believe, in the rate of injection. Thus in experiment I, the lapse of time from the beginning of the injection to the time the threshold of the kidney was reached was forty-nine minutes (3.25 to 4.14 P. M.) ; in experiment II, this interval was twenty-three minutes (4.02 to 4.25 P. M.), and in experiment III, it was nine minutes (2.35 to 2.44 P. M.).

It is suggested at once that even while we are injecting, the hemoglobin is being removed at a definite rate by the tissues (liver?) as well as by the kidney, and that when the threshold value is reached we have actually less hemoglobin present in the serum than the above figures would indicate. Thus, if in experiments II and III (which were on the same animal and are, therefore, comparable) we assume that from the moment the injection began, tissues other than the kidneys were removing the hemoglobin at the rate of 0.054 of a gram per minute, then in experiment II at the end of twenty-three minutes there will have been removed by the tissues 1.24 grams (23 times 0.054 of a gram) of the 1.95 grams injected, leaving 0.71 of a gram (0.06 of a gram per kilo) present in the blood serum at 4.25 P. M. when the kidney threshold was reached; and in experiment III at the end of nine minutes there will have been removed 0.49 of a gram (9 times 0.054 of a gram) of the 1.2 grams injected, leaving again 0.71 of a gram (0.06 of a gram per kilo) present in the blood serum at 2.44 P. M. when the threshold was reached. That this rate is not constant for all animals is shown in experiment I, in which the absorption took place more slowly. That the hemoglobin is nevertheless removed from the serum at about the rate of 0.05 of a gram per minute is borne out by the fact that after the injection of 2.8 to 4 grams of hemoglobin, we found the serum almost clear in about an hour or a little more. We therefore conclude that elimination of hemoglobin by the kidneys does not occur until it is present in the serum in a concentration of about 0.06 of a gram per kilo of body weight.

Concerning the amount of hemoglobin eliminated by the kidneys,

it is probable that were it possible to stop injecting at the moment the renal threshold is reached, the quantity of hemoglobin eliminated would be a mere trace that could not be estimated. To do this, however, is practically impossible since one must wait for about eight minutes in order to detect the hemoglobin in the urine after the threshold is reached; but if very small amounts of hemoglobin are injected at such long intervals the hemoglobin will be entirely removed by tissues other than the kidneys during the eight minute intervals and the threshold will never be reached.

In each of the experiments quoted it happened that one injection of hemoglobin was given after the renal threshold was reached. Thus in experiment I, at 4.21 P. M., 0.32 of a gram of hemoglobin had been injected; in experiment II, at 4.30 P. M., 0.24 of a gram of hemoglobin had been injected; and in experiment III, at 2.49 to 2.51 P. M., 0.39 of a gram of hemoglobin had been injected. The total amounts eliminated in the urine were in experiment I, 0.18 of a gram; in experiment II, 0.05 of a gram; and in experiment III, 0.15 of a gram (table I). Thus these quantities bear, as we should expect, a relation to the amount of blood injected above the threshold value; the amount eliminated being about half (or somewhat less), in excess over the threshold value. There is, of course, no relation between the total amount injected and the amount recovered in the urine, nor should we expect such a relation since the kidney is active only when a concentration has been reached which is above the threshold value for the kidney.

In none of these experiments was jaundice observed, and, as a matter of fact, we have found that in all normal animals, when injections are made slowly, jaundice is less readily produced than when the injections are made rapidly, as in the experiments to be described later.

The next problem for consideration was that concerning the degree of retention of hemoglobin necessary to cause jaundice. In order to study this question quantitatively and accurately in the shortest possible time, decreasing quantities of hemolyzed blood were injected intravenously into a series of normal dogs; in each case the percentage elimination by the kidney and occurrence or non-occurrence of bile pigments in the urine were noted.

TABLE I.
Details of Injection of Hemoglobin.

Experiment No.	Remarks.	Injection time.		Hemoglobin injected.		Hemoglobin eliminated.		Hemoglobin retained.			Choluria.
		Duration in min.	Gm. of hemoglobin per kilo per min.	Total in gm.	Amount in gm. per kilo.	Total in gm.	Per cent.	Total in gm.	Amount in gm. per kilo.	Per cent.	
I	Normal	56	0.006	2.91	0.35	0.18	6.1	2.73	0.33	93.9	None.
II	Normal	28	0.007	2.19	0.20	0.05	2.3	2.14	0.19	97.7	None.
III	Normal	16	0.009	1.60	0.14	0.15	9.6	1.45	0.13	90.4	None.
VI	Normal	13	0.045	3.36	0.56	1.09	32.5	2.27	0.39	67.5	Marked.
VII	Normal	8	0.039	2.38	0.31	0.64	26.8	1.74	0.23	73.2	Faint trace.
VIII	Normal	11	0.024	2.21	0.26	0.38	17.1	1.83	0.22	82.9	Very faint trace.
IX	Normal	4	0.072	2.03	0.29	0.73	35.9	1.30	0.18	64.1	None.
X	Before splenectomy	27	0.030	5.03	0.81	0.85	16.0	4.18	0.68	84.0	Marked.
X	After splenectomy	40	0.013	3.36	0.54	0.63	18.8	2.73	0.44	81.2	Marked.
XI	Before splenectomy	32	0.012	3.94	0.38	1.06	26.5	2.88	0.28	73.5	Faint trace.
XI	After splenectomy	8	0.046	3.92	0.37	0.66	16.8	3.26	0.31	83.2	Faint trace.
XII	Splenectomy 28 days	10	0.032	3.08	0.32	0.65	21.1	2.43	0.25	78.9	Present.
XIII	Splenectomy 9 mos.	10	0.038	6.57	0.38	1.81	27.5	4.76	0.28	72.5	Trace.
XIV	Splenectomy 61 days	5	0.056	2.09	0.28	0.15	7.2	1.94	0.26	92.9	Present.
XV	Splenectomy 60 days	7	0.039	3.46	0.27	0.64	18.4	2.82	0.22	81.6	Trace.
XVI	Splenectomy 110 days Hemolytic jaundice	10	0.025	3.92	0.25	1.12	28.6	2.80	0.18	71.4	Marked.
XVII	Splenectomy 23 days Obstructive jaundice	7	0.036	2.23	0.25	0.59	26.4	1.67	0.19	73.6	Marked.

The following experiments are illustrative.

Experiment VI.—Normal dog, female; weight 5,780 gm. Ether anesthesia; cannula in small branch of saphenous vein; catheter in bladder; 300 c.c. of water by stomach tube.

March 27, 1912. 3.20 P. M. Urine normal. Blood serum clear.

3.32-3.45 P. M. 150 c.c. of hemoglobin solution (3.36 gm. of hemoglobin) injected.

3.47 P. M. Blood serum deeply stained with hemoglobin.

3.48 P. M. Urine contained hemoglobin in small amount.

3.49 P. M. Urine deeply stained with hemoglobin.

12.00 M. Urine collected at this time contained 1.09 gm. of hemoglobin. Bile pigment present.

March 28, 1912. 8.00 A. M. The cage urine contained much bile pigment; no hemoglobin, no albumin.

10.00 A. M. Urine obtained by catheter contained bile pigment in lessened amount.

March 29, 1912. Faint trace of bile pigment in urine.

Summary.—The total amount of hemoglobin injected was 3.36 gm. (0.58 gm. per kilo). The total amount eliminated by the kidney was 1.09 gm. The amount retained was, therefore, $3.36 - 1.09 \text{ gm.} = 2.27 \text{ gm.}$ (0.39 gm. per kilo). Bile pigments appeared in the urine in considerable amounts.

Experiment VII.—Female dog; weight 7,700 gm. Ether anesthesia. Injections of hemoglobin solution through hypodermic needle into superficial vein of lower part of leg.

March 28, 1912. 2.00 P. M. No albumin and no bile in urine.

3.59-4.07 P. M. Injection into vein of 96.5 c.c. of hemoglobin solution (2.38 gm. of hemoglobin).

4.12 P. M. Catheterized. Hemoglobinuria was marked.

March 29, 1912. The cage urine contained much hemoglobin, a trace of bile, but no albumin. Total hemoglobin eliminated = 0.64 gm.

10.30 A. M. Urine obtained by catheter contained no hemoglobin, and no albumin, but a trace of bile.

March 30, 1912. No albumin and no bile in the urine.

Summary.—The total amount of hemoglobin injected was 2.38 gm. (0.31 gm. per kilo). The total amount eliminated by the kidney was 0.64 gm. The amount retained was, therefore, 1.74 gm. (0.23 gm. per kilo). For about twenty-four hours bile pigments occurred in traces in the urine.

Experiment VIII.—Female dog; weight 8,435 gm. Ether anesthesia. Injections through hypodermic needle into superficial vein of lower part of leg.

March 29, 1912. No bile in urine.

3.38-3.49 P. M. Injection into vein of 90 c.c. of hemoglobin solution (.21 gm. of hemoglobin).

11.50 P. M. The cage urine (31 c.c.) contained 0.38 gm. of hemoglobin. There was a light cloud of albumin and the faintest possible trace of bile.

March 30, 1912. 10.00 A. M. The cage urine contained no hemoglobin, and no bile, but a trace of albumin.

March 31, 1912. The cage urine contained no bile.

Summary.—The total amount of hemoglobin injected was 2.21 gm. (0.26 gm. per kilo). The total amount eliminated by the kidney was 0.38 gm. The amount retained was, therefore, 1.83 gm. (0.22 gm. per kilo). Bile pigments occurred in the urine for about eight hours in the faintest possible traces.

Experiment IX.—The same dog as in experiment VII; weight 7,010 gm. Ether anesthesia. Injections through hypodermic needle into superficial vein of lower part of leg.

April 2, 1912. No albumin and no bile in urine.

2.50–2.54 P. M. Injection into vein of 71.5 c.c. of hemoglobin solution (2.03 gm. of hemoglobin).

April 3, 1912. 8.00 A. M. Urine contained 0.73 gm. of hemoglobin, no bile, and no albumin.

April 4, 1912. Urine contained no bile and no albumin.

Summary.—The total amount of hemoglobin injected was 2.03 gm. (0.29 gm. per kilo). The total amount eliminated by the kidney was 0.73 gm. The amount retained was, therefore, 1.30 gm. (0.18 gm. per kilo). No bile pigments appeared in the urine.

A comparison of these four experiments on normal dogs shows that the retention of 0.39 of a gram of hemoglobin per kilo caused marked choluria; of 0.23 of a gram, slight choluria for twenty-four hours; of 0.22 of a gram, a very faint choluria for eight hours; and of 0.18 of a gram, no choluria. The threshold for jaundice by this method in the normal dog lies apparently, therefore, between about 0.18 of a gram and 0.22 of a gram of hemoglobin per kilo of body weight.

The percentage of hemoglobin eliminated by the kidney appears to be a variable quantity. Thus, in experiment VI, 32.5 per cent. of the hemoglobin injected was eliminated by the kidney; in experiment VII, 26.8 per cent.; in experiment VIII, 17.1 per cent.; in experiment IX, 35.9 per cent.

In these four experiments the hemoglobin solution was rapidly injected during a period of from four to thirteen minutes. When the solution was introduced slowly a much larger amount could apparently be cared for in the liver without the production of jaundice. Thus, if we refer again to experiment I, in which the solution was introduced at intervals throughout a period of fifty-six minutes, we find that an amount of hemoglobin was retained equal to 0.33 of a gram per kilo, without bile pigments occurring in the urine.

DISCUSSION.

These experiments seem definitely to establish the mechanism by which free hemoglobin is removed from the blood serum under normal conditions. Our conception of this mechanism is as follows. The kidney does not eliminate hemoglobin until its concentration in the blood serum reaches a certain level. This concentration we conclude from experiments I, II, and III is about that produced by the presence of 0.06 of a gram of free hemoglobin per kilo of body weight. As soon as the concentration of the hemoglobin in the serum is above this point, the hemoglobin passes through the kidneys and we have hemoglobinuria, but as soon as it falls below this amount, the hemoglobinuria ceases. However, other tissues, of which presumably the liver is the most important, appear to take up hemoglobin as soon as mere traces are present in the serum and continue to remove it from the serum whether the renal threshold is exceeded or not. Therefore, whenever the kidney is removing hemoglobin from the serum these other tissues are also removing it. The kidneys apparently remove 17 to 36 per cent., and the liver (and other tissues?) 64 to 83 per cent.

The hemoglobin which the liver removes is changed into bile pigment, which, if it is not produced in too large amounts, or if the hemoglobin is not taken to the liver too rapidly, passes out as bile pigment in the usual manner through the bile passages. On the other hand, if the hemoglobin is taken up by the liver in larger quantities, and especially if this occurs rapidly, the bile is formed faster than the bile capillaries can remove it and it is reabsorbed into the circulation and appears in the urine. Obviously if this hypothesis is correct, the larger the amount of bile pigment already present in the liver, the smaller will be the amount of hemoglobin necessary to call forth a choluria, and *vice versa*.

Whether the accumulation of bile pigment or of hemoglobin in the liver influences the rate at which the liver removes hemoglobin from the serum has not been determined with certainty, but some of the experiments (to be presented later) apparently show a greater elimination of the hemoglobin by the kidney when the liver is presumably more or less saturated, as in spontaneous and obstructive jaundice, and the natural explanation for this is, that accumulation

in the liver diminishes the rate at which the liver takes up the hemoglobin from the serum, and, consequently, more hemoglobin is left for the kidneys to remove.

The Effect of Splenectomy.—Having determined these facts concerning the elimination of hemoglobin and the production of jaundice in normal dogs, we next studied the effect of splenectomy.

In two experiments hemoglobin solution was injected into normal dogs, after which these animals were splenectomized and later the hemoglobin injections were repeated.

The experiments follow.

Experiment X.—Normal dog, female; weight 6,190 gm. Ether anesthesia; cannula in small vein of left leg; catheter in bladder; 300 c.c. of water by stomach tube.

Feb. 26, 1912. 3.25 P. M. Urine normal.

3.28–3.55 P. M. 140 c.c. of hemolyzed blood injected (5.03 gm. of hemoglobin).

4.10 P. M. Urine contained hemoglobin.

Feb. 27, 1912. 1.00 A. M. Urine contained much hemoglobin (0.80 gm. of hemoglobin). No bile pigment.

8.13 A. M. Urine obtained by catheter contained a small amount of hemoglobin (0.05 gm.). Faint but definite positive test for bile pigment.

3.00 P. M. Urine free of hemoglobin; bile test strongly positive.

4.00 P. M. Splenectomy.

Feb. 28, 1912. Moderate amount of bile pigment in urine.

Feb. 29, 1912. Trace of bile pigment in urine. Animal prepared for test as on Feb. 26. Injection into small vein of right leg.

2.30–2.42 P. M. Faint trace of bile pigment.

2.42–3.22 P. M. Slow injection of 140 c.c. of hemolyzed blood (3.36 gm. of hemoglobin).

3.05 P. M. Urine contained hemoglobin (at this time, 2.49 gm. of hemoglobin had been injected).

3.42 P. M. Urine contained much hemoglobin.

March 1, 1912. 8 A. M. The cage urine was slightly tinged with hemoglobin. The total amount eliminated was 0.63 gm. There was a faint bile reaction.

9.00 A. M. Urine obtained by catheter was free from hemoglobin, but gave a well marked bile reaction.

Summary.—Before splenectomy the amount of hemoglobin injected was 5.03 gm. (0.81 gm. per kilo), the amount eliminated by the kidneys was 0.85 gm., and the amount retained was 4.18 gm. (0.68 gm. per kilo). Bile pigment was abundant in the urine. The hemoglobin eliminated through the kidneys was 16 per cent. of the total amount injected. After splenectomy the amount of hemoglobin injected was 3.36 gm. (0.54 gm. per kilo); the amount eliminated by the kidneys was 0.63 gm., and the amount retained was 2.73 gm. (0.44 gm. per kilo). Bile pigment was abundant in the urine. The percentage of hemoglobin eliminated through the kidneys was 18.8 per cent. of the total amount injected.

In the second experiment of the same type smaller amounts of hemoglobin were used.

Experiment XI.—Normal dog, female; weight 10,500 gm. Ether anesthesia; catheter in bladder; cannula in small vein of left leg; 300 c.c. of water by stomach tube.

March 4, 1912. 4.10 P. M. Urine normal.

4.18–4.23 P. M. 50 c.c. of hemolyzed blood injected (1.97 gm. of hemoglobin).

4.45–4.50 P. M. 50 c.c. of hemolyzed blood injected (1.97 gm. of hemoglobin).

4.55 P. M. Urine contained 0.11 gm. of hemoglobin.

March 5, 1912. 8.00 A. M. The cage urine contained 0.95 gm. of hemoglobin. Bile test was faintly positive.

5 P. M. Urine obtained by catheter contained no hemoglobin and no bile.

March 6, 1912. 2.00 P. M. Urine obtained by catheter contained no hemoglobin, but had a very faint trace of bile.

4.00 P. M. Splenectomy.

March 8, 1912. The animal was prepared for test as on March 4; injection into small vein of right leg.

4.30 P. M. Urine normal.

4.32–4.40 P. M. Injection of 100 c.c. of hemolyzed blood (3.92 gm. of hemoglobin).

4.47 P. M. Urine contained hemoglobin.

March 9, 1912. Urine contained much hemoglobin and a trace of bile.

The total elimination of hemoglobin was 0.66 gm.

March 10–11, 1912. Urine showed faint trace of bile.

March 12–13, 1912. Urine free of bile.

Summary.—Before splenectomy the amount of hemoglobin injected was 3.94 gm. (0.38 gm. per kilo). The amount eliminated by the kidneys was 1.06 gm., and the amount retained was 2.88 gm. (0.28 gm. per kilo). Bile pigments appeared in the urine in traces. The percentage of hemoglobin eliminated through the kidneys was 26.5. After splenectomy the amount of hemoglobin injected was 3.92 gm. (0.37 gm. per kilo), the amount eliminated by the kidneys was 0.66 gm., and the amount retained was 3.26 gm. (0.31 gm. per kilo). Bile pigments were present in the urine in traces. The percentage of hemoglobin eliminated through the kidneys was 16.8 per cent.

In this experiment, therefore, the spleen did not have any influence on the elimination or the retention of hemoglobin, or on the retardation of the appearance of bile pigments in the urine.¹ However, as these experiments were done only a few days after splenectomy and with quantities of hemoglobin larger than the minimum

¹ Recently it has been shown by Gilbert, A., Chabrol, E., and Bénard, H. (*Recherches sur la biligénie consécutive aux injections expérimentales d'hémoglobine, Presse méd.*, 1912, xx, 113) that absence of the spleen does not influence the power of the liver to transform into bile the hemoglobin furnished in the form of laked blood.

necessary to produce jaundice in normal animals, it was thought wise to make the same test at longer periods after operation and with smaller quantities of hemoglobin.

Experiment XII.—Female dog.

March 13, 1912. Splenectomy.

April 10, 1912. Weight 9,740 gm. Ether anesthesia; needle in small vein of leg. No albumin and no bile in urine.

4.04–4.10 P. M. Injected 76 c.c. of a solution containing 2.66 gm. of hemoglobin.

April 11, 1912. 8.00 A. M. The cage urine contained 0.15 gm. of hemoglobin and also a quantity of precipitated and altered hemoglobin that could not be accurately measured; no bile pigment was present.

10.00 A. M. Urine obtained by catheter contained no hemoglobin and no bile.

4.00–4.10 P. M. Injected 122 c.c. of a solution containing 3.08 gm. of hemoglobin.

April 12, 1912. 8.00 A. M. The cage urine contained 0.65 gm. of hemoglobin, a trace of bile, and a light cloud of albumin.

9.30 A. M. Urine obtained by catheter showed moderate bile test; no albumin.

April 13, 1912. No bile in urine.

Summary.—At the first injection, 2.66 gm. of hemoglobin (0.27 gm. per kilo) were introduced; the amount eliminated by the kidneys was uncertain; no bile pigment appeared in the urine. A second injection of 3.08 gm. of hemoglobin (0.32 gm. per kilo) was made one day later. The amount eliminated by the kidneys was 0.65 gm., and the amount retained was 2.43 gm. (0.25 gm. per kilo). Bile pigment was present in the urine. The amount of hemoglobin eliminated by the kidneys was 21.1 per cent.

Experiment XIII.—July 8, 1911. Splenectomy.

April 2, 1912. (Nine months after splenectomy.) Weight 17,220 gm. Ether anesthesia; needle in small vein of leg. Urine contained no albumin and no bile.

3.17–3.27 P. M. Injected 167.5 c.c. of solution containing 6.57 gm. of hemoglobin.

April 3, 1912. 8.00 A. M. The cage urine contained 1.81 gm. of hemoglobin and had a faint trace of bile.

April 4, 1912. Urine contained no hemoglobin, no albumin, and no bile.

Summary.—The amount of hemoglobin injected was 6.57 gm. (0.38 gm. per kilo), the amount eliminated by the kidneys was 1.81 gm., and the amount retained was 4.76 gm. (0.28 gm. per kilo). Bile pigments were present in the urine in traces. The hemoglobin eliminated by the kidneys equalled 27.5 per cent. of the total amount injected.

Experiment XIV.—February 10, 1912. Splenectomy.

April 11, 1912. Weight of animal 7,520 gm. (Sixty-one days after splenectomy.) Ether anesthesia; needle in small vein of leg.

Urine contained no albumin and no bile.

3.33–3.38 P. M. Injected 59.8 c.c. of a solution containing 2.09 gm. of hemoglobin.

7.20 P. M. The cage urine contained 0.15 gm. of hemoglobin, no albumin, and no bile.

April 12, 1912. 8.00 A. M. The cage urine was free from hemoglobin; bile pigment present; no albumin.

9.30 A. M. Urine obtained by catheter showed distinct reaction for bile.

April 13, 1912. No bile.

Summary.—The amount of hemoglobin injected was 2.09 gm. (0.28 gm. per kilo), the amount eliminated by the kidneys was 0.15 gm., and the amount retained was 1.94 gm. (0.26 gm. per kilo). Bile pigments were present in the urine in considerable amount. The hemoglobin eliminated by the kidney equalled 7.2 per cent. of the total amount injected.

Experiment XV.—February 10, 1912. Splenectomy.

April 10, 1912. Weight 12,680 gm. (Sixty days after splenectomy.) Ether anesthesia; needle in small vein of leg.

No albumin and no bile in urine.

4.22–4.29 P. M. Injected 99 c.c. of solution containing 3.46 gm. of hemoglobin.

April 11, 1912. 8.45 A. M. The cage urine contained 0.36 gm. of hemoglobin in solution and about 0.28 gm. of precipitated hemoglobin; the total amount eliminated was about 0.64 gm. No albumin; faint trace of bile.

9.30 A. M. Urine obtained by catheter contained a trace of bile; no hemoglobin.

April 12, 1912. No bile.

Summary.—The amount of hemoglobin injected equalled 3.46 gm. (0.27 gm. per kilo), the amount eliminated by the kidneys was about 0.64 gm., and the amount retained was about 2.82 gm. (0.22 gm. per kilo). A trace of bile appeared in the urine. The amount of hemoglobin eliminated by the kidneys was about 18.4 per cent. of the total amount injected.

These six experiments on splenectomized animals, in all of which bile pigments appeared in the urine for a short time and in small quantities after the retention of 0.44, 0.31, 0.25, 0.28, 0.26, and 0.22 gm. per kilo, respectively, indicate that the threshold for jaundice in splenectomized dogs is approximately 0.22 of a gram per kilo, the same as in the experiments (VI to XI) with normal dogs, in which the threshold was found to be between 0.18 and 0.22 of a gram per kilo.

When we examine the percentage of hemoglobin eliminated by the kidneys in the six splenectomized animals, we find that it runs a trifle lower than the limits determined for normal animals, being 18.8, 16.8, 21.1, 27.5, 7.2 (?), and 18.4 per cent. (average, excluding the fifth figure, 20.5 per cent.), as compared with 32.5, 26.8, 17.1, 35.9, 16, and 26.5 per cent. (experiments VI to XI), with an average of 25.8 per cent. We can, therefore, conclude that splen-

ectomy has no influence in increasing the elimination of free hemoglobin by the kidneys nor does it, as is shown by the occurrence of choluria in each of the experiments, alter the ability of the liver to form bile pigments from hemoglobin, or interfere with the elimination of these pigments. Thus one of the possible explanations for the failure of jaundice to follow the administration of a hemolytic serum in splenectomized animals, as suggested in the first paper² of this series, is shown to be untenable.

Our next experiments were undertaken for the purpose of determining whether a liver saturated with bile pigments would take up hemoglobin from the serum less rapidly than a normal liver and thus lead to the elimination of a larger percentage of hemoglobin through the kidneys. For this purpose we used (1) a dog that, after splenectomy, had developed a spontaneous choluria, and (2) a dog with obstructive jaundice. The observations on the former follow.

Experiment XVI.—November 15, 1911. Splenectomy.

March 4, 1912. Weight of animal 15,550 gm. (110 days after splenectomy). Ether anesthesia; needle in small vein of leg.

Urine intensely stained with bile pigment.

Injection during a period of ten minutes of 104 c.c. of a solution containing 3.92 gm. of hemoglobin.

March 5, 1912. 8 A. M. The cage urine contained 1.12 gm. of hemoglobin. Urine was deeply bile-stained.

5.00 P. M. The cage urine was free from hemoglobin, but bile pigment was still abundant.

Summary.—The amount of hemoglobin injected equalled 3.92 gm. (0.25 gm. per kilo), the amount eliminated by the kidneys was 1.12 gm., and the amount retained was 2.80 gm. (0.18 gm. per kilo). Bile pigments continued to be as abundant as before the experiment. The hemoglobin eliminated by the kidneys equalled 28.6 per cent. of the total amount injected.

The second experiment was on a splenectomized dog in which we ligated the common bile duct two days before the injection of the hemoglobin.

Experiment XVII.—Female dog.

March 6, 1912. Splenectomy.

March 27, 1912. Common bile duct doubly ligated. Severed between ligatures.

March 28, 1912. Large amount of bile in urine.

² Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, *Jour. Exper. Med.*, 1912, xvi, 363.

March 29, 1912. Much bile and no albumin in urine. Weight 8,945 gm.

3.17-3.24 P. M. Ether anesthesia; needle in small vein of leg. Injected 90.5 c.c. of solution containing 2.23 gm. of hemoglobin.

11.50 P. M. The cage urine contained 0.59 gm. of hemoglobin; trace of albumin; intense bile reaction.

March 30, 1912. The cage urine contained no hemoglobin; trace of albumin; intense bile reaction.

March 31, 1912. Urine the same as on March 30.

Summary.—The amount of hemoglobin injected equalled 2.23 gm. (0.25 gm. per kilo), the amount eliminated was 0.59 gm., and the amount retained was 1.67 gm. (0.19 gm. per kilo). Bile pigments continued to be abundant in the urine. The hemoglobin eliminated by the kidneys equalled 26.4 per cent. of the total amount injected.

The percentage of hemoglobin eliminated by the kidney in these two experiments, namely 28.6 and 26.4 per cent., is distinctly higher than in the six other experiments on splenectomized dogs (with the single exception of experiment XIII), although well within the limits established for normal dogs. With the increased elimination, there was a corresponding slight decrease in the amount retained, as compared with both normal and splenectomized dogs. We conclude, therefore, that saturation of the liver with bile pigments may diminish, but only to a very slight extent, the relative amount of hemoglobin removed from the serum by the liver with a concomitant very slight increase in the amount removed by the kidneys.

SUMMARY.

The results of this study may be stated as follows.

1. Rapid injection of more than 0.06 of a gram per kilo of hemoglobin intravenously into a normal animal is followed by the appearance of hemoglobin in the urine (pelvis of kidney) within eight to ten minutes.
2. After rapid injection of more than 0.012 of a gram per kilo per minute of hemoglobin, 16 to 36 per cent. of the total amount, if this equals 0.25 of a gram per kilo, is eliminated in the urine and is accompanied by choluria.
3. If the injection of not more than 0.35 of a gram per kilo is made slowly (less than 0.01 of a gram per kilo per minute), the amount eliminated in the urine is only 2.33 to 9.5 per cent. of the total amount injected, and choluria does not occur.

4. The concentration of free hemoglobin in the blood which constitutes the threshold value of the kidneys for hemoglobin is approximately 0.06 of a gram of hemoglobin per kilo of body weight. When about this concentration is reached, hemoglobin appears in the urine.

5. The amount of hemoglobin per kilo of body weight which, after rapid injection, may be retained without jaundice, is approximately 0.18 of a gram. When 0.22 or 0.23 of a gram is retained bile pigments appear in the urine. The threshold of the liver for jaundice in point of hemoglobin saturation lies, therefore, between 0.18 and 0.22 of a gram per kilo of body weight. With slow injections a greater amount may be retained without choluria.

6. The absence of the spleen does not alter greatly the percentage of hemoglobin eliminated by the kidney, nor does it raise the threshold of the liver for jaundice.

7. In the presence of jaundice, either hemolytic or obstructive, the amount of hemoglobin retained by splenectomized animals is slightly diminished and that eliminated by the kidneys is correspondingly increased.

Upon these data may be based the following explanation of the mechanism by which free hemoglobin is removed from the blood serum. Hemoglobin is not removed by the kidney until its concentration in the blood serum reaches a certain level (0.06 of a gram of free hemoglobin per kilo of body weight). This constitutes the threshold value of the kidneys for hemoglobin and when it is reached hemoglobin appears in the urine. When the concentration is lower, hemoglobinuria ceases; at the same time, however, the liver, and possibly other tissues, take up hemoglobin as soon as mere traces are present in the serum and they continue this removal whether the renal threshold is exceeded or not. The two processes go on simultaneously, the rate of removal, when the renal threshold is exceeded, being for the kidneys 17 to 36 per cent., and for the liver and other tissues 64 to 83 per cent, of the total amount introduced. The hemoglobin which is removed by the liver is transformed into bile pigments. If the amount reaching the liver is small and is received slowly, the amount of bile formed is not increased above the excretory capacity of the liver, and it is removed by the bile passages

without the occurrence of choluria. This is shown in our experiments in which injections of hemoglobin were made more slowly than 0.01 of a gram per kilo per minute. On the other hand, if the hemoglobin is taken up by the liver rapidly and in large amounts, the bile capillaries are overtaxed and the bile cannot be rapidly removed, but is reabsorbed into the blood, and choluria develops.

If this theory is correct we have an explanation of those instances of blood destruction in man characterized by jaundice, but not accompanied by hemoglobinuria. In a slow, gradual destruction of the red blood cells, the liver removes the hemoglobin from the serum so rapidly that the concentration of hemoglobin in the serum does not reach the threshold value of the kidneys and hemoglobinuria, therefore, cannot occur. The constant absorption of large amounts of hemoglobin by the liver and the increase in bile formation which results does, however, overtax the bile passages and jaundice occurs.

In the same way may be explained the continuance of jaundice after the disappearance of a transient hemoglobinuria. A rapid destruction of a large amount of blood raises the concentration of hemoglobin in the serum so quickly that the threshold value of the kidney is quickly exceeded and hemoglobin appears in large amounts in the urine. When an amount of hemoglobin sufficient to reduce the concentration of the serum below the threshold value of the kidney has been removed, a considerable amount of hemoglobin may still remain in the serum, and it is the slow elimination of this through the liver that causes the choluria to continue.

The demonstration that the absence of the spleen has no important influence on the elimination of hemoglobin by the kidney, on its transformation into bile pigments, or on the removal of such pigments, is of interest in connection with an observation made in the first paper of this series. This was concerning the frequent failure of jaundice to follow the administration of hemolytic serum during the early period following splenectomy.³ Among the possible explanations was the suggestion that the spleen is in some way concerned in the disintegration of free hemoglobin or in the elaboration of its derivatives. The present investigations demonstrate that such an explanation is without experimental basis, though it does not

³ Pearce, R. M., Austin, J. H., and Krumbhaar, E. B., *loc. cit.*

controvert the possibility of the spleen being concerned in liberating hemoglobin from the red cells and suggests that the failure of jaundice is due to some other factor or factors. Evidence to indicate that the changes in the blood that follow splenectomy are important factors is offered in the third paper⁴ of this series.

⁴Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., III. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, *Jour. Exper. Med.*, 1912, xvi, in press.

EXPERIMENTELLE LÄSIONEN DES CENTRAL- NERVENSYSTEMS, UNTERSUCHT MIT HILFE DER VITALEN FÄRBUNG.*

VON DR. JOHN T. MACCURDY UND DR. HERBERT M. EVANS.

*(Aus den Laboratorien des anatomischen und des anatomisch-pathologischen
Institutes der Johns Hopkins-University, Baltimore.)*

Es ist bisher schwer gewesen, durch die gewöhnlichen histologischen Methoden leichte Zellschädigungen oder den eben eingetretenen Zelltod innerhalb der Organe des tierischen Körpers festzustellen. Mit anderen Worten, es musste eine sehr schwere Veränderung der Zelle Platz greifen, ehe man eine Störung des Zellebens mit Sicherheit feststellen konnte. Das zuerst aus dem Aussehen der Zelle auffindbare Symptom einer Schädigung ist unter dem Namen „trübe Schwellung“ bekannt; aber diese Veränderung ist nicht so typisch und so konstant in ihrem Auftreten, um sie zu einem allgemeinen Kriterium der Zellschädigung erheben zu können. Ja sogar den Zelltod können wir erst nach einem längeren Zeitraum wahrnehmen, wie uns die Erfahrung der Pathologen zeigt. Es muss also genügend Zeit vergehen, bis die Degeneration so weit vorgeschritten ist, dass die Färbbarkeit oder die anatomische Struktur der Zellen verändert und uns damit erkennbar wird.

Durch die Arbeiten der letzten Jahre haben wir erfahren, dass es möglich ist, relativ grosse Dosen gewisser Benzidinfarben in den lebenden Körper zu injizieren ohne toxischen Effekt. So lernten wir eine neue Methode kennen, durch die es möglich ist, Zellschädigungen zu erkennen. Die sogenannten „Vitalfarben“ verdanken chemotherapeutischen Experimenten ihre Entdeckung. Diese wurden vor allem von Ehrlich (1905, 1907) und Nicolle und Mesnil (1906) ausgeführt; Bouffard (1906) und besonders Goldmann (1909–1912) haben die Histologie der so vital gefärbten Tiere studiert. Goldmann kommt das Verdienst zu, durch seine

* Mit Unterstützung des Rockefeller Institute for Medical Research, New York.

ausgedehnten Studien am gesunden und kranken Tier den hohen Wert der neuen Methode gezeigt zu haben. Vor allem hat er uns mit der Tatsache bekannt gemacht, dass die angewendeten Farben in Granulaform im Cytoplasma bestimmter Zellen auftreten, die für jedes Organ charakteristisch sind. Unter keinen Umständen tritt eine Kernfärbung lebender Zellen auf, wenn Vitalfarben genannter Klasse benutzt sind).¹⁾

Die Kernfärbung ist also ein wichtiges Kennzeichen für den Zelltod. Andererseits können geschädigte Zellen durch eine diffuse statt der gewöhnlichen Granulafärbung des Cytoplasmas erkannt werden (Goldmann) oder durch eine deutliche Veränderung der Grösse und Zahl der gefärbten Granula (wie Gross [1911] gezeigt hat).

Alle die Erscheinungen sind, auf das Centralnervensystem angewendet, von grösstem Interesse. Normale Nervenzellen färben sich durch die Vitalfarben der Benzidinreihe weder im Protoplasma noch im Kern, so dass das Auftreten der Farbe in der Zelle überhaupt bereits klar einen veränderten Zustand der Zelle zeigt. Wir haben nun durch das Studium normaler und kranker Nervenzellen erwiesen, dass auch diese Zellen in charakteristischer Weise mit den Vitalfarben reagieren. Durch die Güte von Herrn Prof. Simon Flexner, Direktor des Rockefeller Institute for Medical Research, hatten wir Gelegenheit, während des vergangenen Winters an Tieren des Institutes Versuche mit experimenteller Poliomyelitis an Affen anstellen zu können, wobei wir durch die grosse Erfahrung des Laboratoriums liebenswürdig unterstützt wurden. Die Arbeit, über die wir hier berichten, war im Februar dieses Jahres beendet.

Das Studium dieser Krankheit mit Hilfe der Vitalfarbe Trypanblau hat klar gezeigt:

1. Die frisch getöteten Nervenzellen sind spezifisch durch die Vitalfarbe gefärbt. Neben der Kernfärbung findet sich eine diffuse Protoplasmafärbung. Ebenso sind alle dendritischen Fortsätze gefärbt, die sich scharf von ihrer ungefärbten Umgebung abheben.

¹⁾Ribbert und Schlecht machten mit Lithionkarmin, das intravenös injiziert wurde, die gleiche Erfahrung.—Rost ist bei seinen Arbeiten mit Methylenblau und Neutralrot zu einem ähnlichen Schluss gekommen (vgl. Rost, Pflüger's Archiv, Bd. 137).

2. Die geschädigten Nervenzellen sind an dem Auftreten der Farbe in Granulaform im Protoplasma zu erkennen.

Ferner untersuchten wir die bei der Einimpfung des Virus in das Gehirn unvermeidbare Wunde. Das Studium derselben zeigte, dass

1. getötete Gliazellen ebenso wie Nervenzellen eine allgemeine Färbung zeigen, d. h. ein helles diffuses Cytoplasma und eine tiefere Kernfärbung aufweisen;

2. die in der Pathologie des Centralnervensystems so wohl-bekannten Körnchenzellen prachtvoll vitalgefärbt sind, d. h. leuchtende Farbgranula in ihrem Protoplasma beherbergen. Viele dieser Zellen befinden sich in Mitose, viele sind auch mit Fett beladen. Andere vitalgefärbte Zellen liegen im Wundsekret und ähneln Makrophagen;

3. die Endothelzellen der der Wunde benachbarten Blutgefässcapillaren des Gehirns vitalgefärbte Granula im Protoplasma enthalten.

Wir hoffen bald in extenso über unsere bereits weiter fortgesetzten Studien auf diesem Gebiet zu berichten und vor allem zu zeigen, welches Licht die Vitalfärbung auf gewisse histogenetische Vorgänge innerhalb des Centralnervensystems wirft.

LITERATUR.

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TRANSPLANTABILITY OF MALIGNANT TUMORS TO THE EMBRYOS OF A FOREIGN SPECIES.

By JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

It appeared early in the development of modern surgery that tissues of lower animals could not be grafted into human beings. The extent of this barrier between species was not fully appreciated until the discovery of the transplantability of tumors. It was soon demonstrated by experiment that tumors of lower animals not only fail to grow when inoculated into another species, but often fail or give only slight growth, when grafted into a different variety of the same species. For example, the tumors of the white mice usually fail to "take" when inoculated into the gray or brown mouse. Recently Rous¹ has reported a tumor of a pedigreed chicken, from the first inoculations of which tumors resulted in none other than blood-related individuals; and only after the malignancy had become greatly increased by repeated transfer did growth take place in other varieties of chickens.

The idea of the specificity of transplanted tumors has become so firmly established among students of cancer research that the fact of a new growth of any kind proving transferable to another species is considered evidence against the growth being a true neoplasm. The views entertained of genital tumor of the dog transplanted to the fox, and a tumor of the hare successfully inoculated into the rabbit, serve as examples of this.²

Ehrlich³ was the first to demonstrate that while tumors of one species cannot be transferred, in the true sense of the word, to a

1. Rous: Jour. Exper. Med., 1910, xii, 696.

2. Von Dungern and Coca: Ztschr. f. Immunitätsforsch., 1909, ii, 391. Von Dungern: München. med. Wchnschr., 1912, lix, 238.

3. Ehrlich, P.: Arb. a. d. k. Inst. f. exper. Therapie, 1906, No. 1, p. 84.

foreign species, yet such tissue when introduced into a closely related species may survive for a short time (from eight to ten days) and the cells actually multiply. If the tissue during this period is returned to the original species, it seems unaffected by its sojourn in the foreign host and is capable of producing an actively growing tumor. If, however, the graft is allowed to remain, or is transferred to another host of foreign species, it soon dies and is absorbed. These observations are accounted for by Ehrlich on the theory that tumor cells lack proper receptors to combine with the food elements of the foreign species and die from lack of nourishment. He considers that enough specific food is carried along with the graft from the original host to account for the temporary survival and growth. When this supply is exhausted the cells quickly die.

We reported last year⁴ the successful inoculation of the Rous chicken sarcoma into the developing chick embryo. In the course of this study the important observation was made that the tumor grew well in the developing pigeon or duck embryo, whereas in the adults of these species it quickly died and was absorbed. This fact suggested a wide difference between the embryo and the adult in their reaction to foreign tissues. The embryo, we might say, either provides a food substance utilizable by these tissues, which is lacking in the adult, or else lacks a defensive mechanism against such an invasion, which is possessed by the adult. The following results, obtained with tissues of a more distantly related species, constitute another and much more remarkable instance of similar kind.

Following the technic described in former papers,⁴ I inoculated a fine hash of the Jensen rat sarcoma into the outer membrane (fused chorion and allantois) of five-day to seven-day chick embryos; these were then incubated from ten to twelve days, that is, to within two days of hatching. When the shell was removed and the membrane cut and turned back, in a high percentage of instances the egg was found to have, at the point of inoculation, a rounded globular mass, suspended from the membrane by a broad flat pedicle.

4. Murphy, J. B., and Rous, P.: Tumor Implantations in the Developing Embryo; Experiments with a Transmissible Sarcoma of the Fowl, *THE JOURNAL A. M. A.* March 11, 1911, p. 741; *Jour. Exper. Med.*, 1912, xv, 119.

The masses varied in size from 0.2 to over 2 cm. in diameter. The consistency was that of a firm jelly, varying in color from pinkish-gray to red. The surface was smooth and glistening, with numerous large dilated vessels coursing over it, and smaller vessels were visible in the semitranslucent substance of the tumor itself. On section the masses were found to be solid and composed throughout of this pinkish-gray, somewhat translucent tissue, with small areas of hemorrhage in some cases. The tissue was quite moist and friable.

In the rat the Jensen tumor is made up microscopically of characteristic large spindle-cells, with a clear vacuolar nucleus and a fairly deep-staining protoplasm. The cells lie close together, with little stroma, or intercellular substances. Thin-walled blood-vessels are fairly frequent, and occasionally mitotic figures are seen. The cells of the new tumors grown in the chick embryo have an almost identical structure with those of the tumor grown in rats. The cells, however, are not so compact, in some cases forming only a loose network, with clear spaces between. Mitosis is extremely frequent, almost every field showing some stage of this process, and as many as five such figures have been seen in an area taken in by the oil-immersion lens. The blood-vessels filled with nucleated chicken blood-cells are numerous, budding out from the membrane in large tufts. There is practically no stroma, except a slight amount carried with the vessels. The surface of the mass is covered with a continuation of a thin layer from the chick membrane.

The results in the adult chicken are very different. Here the tissue of the Jensen sarcoma is so quickly destroyed that few of its cells remain intact after twenty-four hours. That in the embryo the rat cells retain their biologic characters is shown by the fact that the tissue, when returned to the rat after a sojourn of from ten to twelve days in the chick, causes an actively growing tumor of the Jensen type.

The results obtained up to this stage of the experiment, that is, the active growth of cells in a foreign species over a period of from ten to twelve days, might possibly be accounted for in the same manner that Ehrlich explains the survival and slight growth of the mouse sarcoma in the rat. The results are hardly comparable, how-

ever, when it is considered that in the embryo the cells at the twelfth day are still in a state of active growth, with no indication of degeneration or of a defensive reaction on the part of the host; while in the Ehrlich experiments only the outermost cells survive, and there is a marked defensive reaction on the part of the foreign host. The next experiments remove any doubt as to the untenability of the Ehrlich theory in explaining the case.

It was found that the tissue of the tumors formed in the membranes of the chick by the inoculations with the Jensen rat sarcoma would continue to grow if implanted in another developing chick embryo. The tumors resulting from the second series of inoculations were similar in every respect to those of the first. In like manner a third and fourth successful transfer could be made. This is the stage I have at present reached, with a total of forty-six days of continuous growth of the rat tumor in the chick embryo. The tumors at this stage are similar to those of the original inoculation. The cells retain their form and structure unaltered, are fairly compact, and show numerous mitoses. No degeneration or irregularity of the division figures occurs. Tissues from the second and third transplantation series when returned to the rat caused actively growing tumors. No inoculations were made from the fourth series into rats, as the tissues were needed for other purposes.

It would naturally be expected that after so long a dependence on a foreign food there might be some change in the direction of an adaptation on the part of the rat cells to the new conditions. That this change cannot be marked, however, is shown by the fact that the rat tissue, grown for so long a period in the chick embryo, is almost as quickly killed when introduced into the adult chick as is the tissue taken direct from the rat. There is, perhaps, a slight difference in the reaction on the part of the adult to such inoculations. This point will be discussed in a subsequent paper.

A variety of other tissues have been found to grow quite as well as the Jensen tumor when inoculated into the chick. I have grown in this way, for periods varying from seven to twelve days, chick embryonic tissue, rat embryonic tissue, a sarcoma, a carcinoma and a chondroma of the mouse, and the Flexner-Jobling adenocarcinoma of the rat. The rat embryonic tissue and the mouse sarcoma and

carcinoma were successfully reinoculated into the original species after growth in the foreign embryo.

CONCLUSION.

It is here shown conclusively that mammalian tumor tissue can live and grow actively in the chick embryo, although in the adult chicken it quickly dies and is absorbed. By transfer from one embryo to another the mammalian tissue can be kept growing continuously in the avian host for as long as forty-six days, and probably indefinitely. This proves beyond doubt that the mammalian cells are able to utilize the food supplied by the avian embryo. Whether the phenomenon is dependent solely on this factor, or whether the absence of a defensive mechanism in the embryo plays the more important part is a subject which is being studied and on which a report will be made later.

THE MODE OF INFECTION IN EPIDEMIC POLIOMYELITIS.

By SIMON FLEXNER, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)*

The problem of prevention of infection in epidemic poliomyelitis still confronts us. Progress in the study of poliomyelitis has been made and knowledge gained, but the question of supreme importance is this: Does this knowledge offer a solution of the mode of infection? With all diseases prevention is far better than the most perfect means of cure; with poliomyelitis it is better, first because at present there exists no specific or true curative treatment, and, second and chiefly, because for the most part when the disease is first recognized it has already caused irreparable damage. An understanding of the mode of infection would lead inevitably to the framing of measures of prevention which, with reasonable certainty, could be expected to bring about a limitation of the extent of the epidemic spread.

The infectious agent or virus of poliomyelitis attacks chiefly the central nervous system. Indeed, it has been detected regularly in the spinal cord and brain, and in the mesenteric lymph-nodes among all the internal organs. It has also been detected in the mucous membrane of the nose and throat, and in the mucus secretions of this membrane, and in the mucus secretions of the stomach and the small and large intestine. The virus has not been detected in such important organs as the spleen, kidneys, liver or bone-marrow. The fact is significant, but in attempting to interpret it, account should be taken of the circumstance that at present we possess only one means of detecting the virus, and that consists in its transmission to monkeys, in which it produces characteristic paralyses and anatomic changes. On this account small quantities of the virus may conceivably escape discovery. The conclusion is none the less inevitable, however, that

detectable amounts of the poliomyelitic virus exist only in the few situations and organs mentioned. The distribution of the virus is identical in human beings who are subjects of so-called spontaneous poliomyelitis, and in monkeys in which the experimental affection is produced. Nor does it matter how the experimental inoculation is accomplished and whether the virus is introduced by injection into the brain or large nerves or subcutaneous tissue or peritoneum, or whether it is merely applied to the nasal mucous membrane. In whatever way the infection is produced purposely, the distribution of the virus in the infected monkeys is the same as in infected human beings.

The precise facts of the distribution of the virus are important for another reason than the establishment of the pathology of the disease. The virus is one that is not known to increase aside from the infected body, and hence in order that it shall be capable of propagating poliomyelitis, it must secure a means of escape from the infected animal. The escape is now known to occur along with the secretions of the nose and throat, and the discharges from the intestine. We are obliged therefore to ask ourselves what the means are by which the virus confined within the interior reaches these external surfaces of the body.

Let us begin by disregarding for the moment the essential point of the way in which the virus probably enters the body in infected human beings, and give our attention to the way in which it escapes in the infected monkey into the nose, throat and intestines. We may first consider the instance in which the virus is deposited in the brain, in which it becomes sealed, as it were, and cut off, apparently, from the exterior of the body. Having been injected into the brain tissue, the infectious microorganism constituting the virus multiplies both within and about the brain tissue at the site of inoculation. As multiplication progresses, the virus leaves the original site of injection and wanders to adjacent and distant parts of the central nervous tissues, becoming implanted in the medulla, the spinal cord, the intervertebral ganglia, as well as reaching the pia-arachnoid membranes or meninges, in which it also spreads. Ultimately, when the virus becomes sufficient in amount, it brings about anatomic changes in the nervous system, one of the results of which is

paralysis. The period intervening between the inoculation and the appearance of paralytic symptoms may be as brief as two or three days, or as long as three, four or five weeks. The great disparity in this period depends on the amount and quality of the virus, as well as the degree of resistance of the inoculated monkey.

The virus, which has found its way to the meninges, does not long remain in the cerebrospinal fluid, with which it escapes in part into the blood, in which it does not appear to undergo a further increase in amount, and indeed seems even incapable of surviving long. A part also of the virus contained within the cerebral fluid escapes regularly by way of the lymphatic channels surrounding the short nerves of smell that pass from the olfactory lobes of the brain to the mucous membrane of the nose. It has long been known that there is an intimate connection between the lymphatic vessels of the nasal mucous membrane and the lymphatic spaces of the pia-arachnoidal membrane. Once having gained the mucous membrane of the nose, the virus may even escape into the mucus secretion, with which it is carried into the mouth, and in part swallowed, or it may become established in the substance of the nasal membrane, where it undergoes subsequent multiplication and increase. As a matter of fact both occur. The virus escapes with the secretions partly externally to the infected body, and a part of it is swallowed with the secretions themselves, while a persistent infection of the secretions is maintained by means of the increase that takes place in the membrane itself. In this way is assured the escape of the virus directly into external nature, as well as the contamination of the gastro-intestinal cavity, with the discharges of which it becomes commingled. From the intestine it reaches in some amount the mesenteric lymph-nodes, and thus enables us to account for the occurrence of the virus in those lymphatic nodes which form a notable exception to the general internal organs.

We have now followed the route by which the poliomyelitic virus, implanted within the apparently closed cavity of the skull, reaches the exterior of the body. It is obvious that in the spontaneous form of the infection in man no such mode of introduction of the virus can occur. The virus must indeed enter the human body by some external channel, after which it seeks and becomes implanted on

the central nervous system. It is known that in monkeys the virus is incapable of passing the barrier of the unbroken or slightly abraded skin, or of being taken up from the stomach or intestine unless the functions of these organs are previously disturbed and arrested by opium, and it is further known that it traverses with difficulty or even not at all the substance of the lungs. On the other hand, it is established that the virus passes with readiness and constancy from the intact or practically intact mucous membrane of the nose to the central nervous system, and that this membrane, next to direct intracerebral introduction of the virus, gives the readiest method of successful inoculation.

Hence the view that the nasal mucous membrane is the site both of ingress and egress of the poliomyelitic virus in man. Support for this view is found in the study of the pathology of epidemic poliomyelitis, as well as by the fact established incontestably by experiment that the virus of the disease is regularly present in the nasal mucous membrane and its secretions, both in infected human beings and in monkeys.

Still one link in the chain of causation of epidemic poliomyelitis as here outlined remained to be forged. The clinical evidence is strong in suggesting that human carriers of the poliomyelitic virus exist. The virus has now been detected in the secretions of the nose, throat and intestine of patients suffering from abortive or ambulant attacks of poliomyelitis. The unrecognized examples of the abortive disease play a highly important part in the dissemination of the virus, through which the area of infection is extended, and the number of the attacked increased. Clinical observation accords a similar part to the healthy virus-carrier, who is the last to be detected and confirmed experimentally. Obstacles in the way of this confirmation are considerable, but not insuperable. We possess no means except animal inoculation of discovering the virus of poliomyelitis. Pettersson and his associates now assert that they have demonstrated the virus in washings from the nose and throat of healthy persons in intimate contact with patients having acute cases of the disease.

The preponderance of cases in the late summer and autumn months early suggested an insect carrier of the infection. House-

flies can act as passive contaminators, since the virus survives on the body and within the gullet of these insects. Howard and Clark attempted unsuccessfully to infect the common varieties of mosquito and the body- and head-louse, while they succeeded, in one series, in producing infection in bedbugs which were made to feed on the blood of inoculated monkeys. The virus remained alive within these insects for a period of many days. The inoculation of monkeys with a filtrate prepared from them gave rise to characteristic paralysis and anatomic lesions. This result is significant since it shows that insects are capable of taking up the virus from the blood, in which it exists in minimal quantities, and in harboring it for a considerable period in an active state; but it does not show that multiplication occurs within them or that in nature they act as the agents of inoculation.

It is not permissible to draw hard and fast conclusions regarding the mode of infection in epidemic poliomyelitis from the number of cases occurring, or from their focal distribution, or the seasonal period of the greatest prevalence of the epidemic. While it is true that many more examples of single cases than of multiple cases in a family occur, yet multiple cases are only less frequent than single cases, and not by any means rare. The prevailing views on this topic are being modified rapidly by the recognition of the abortive and ambulant examples of the disease. While the period of greatest prevalence of the epidemic is during the months of August, September and October in the northern hemisphere, and the corresponding months in the southern hemisphere, yet the onset of the epidemic occurs in the early spring and summer months and the disease does not wholly disappear during the winter months. It cannot be affirmed strictly that the disease dies out necessarily at any period of the year. While there are epidemic factors relating to poliomyelitis that are not understood, they are neither remarkable nor unique. If the fact of seasonal prevalence is reversed, all the ordinary factors of case frequency and focal distribution relate equally to epidemic meningitis. Epidemic poliomyelitis, like epidemic meningitis, is preeminently a disease of early childhood, and finds the highest percentage of its victims in the first five years of age, but does not wholly spare older children or even adults.

Like epidemic meningitis, it appears in a frank and in an abortive or ambulatory form, appears to be transmitted by a virus that enters and escapes from the body in the region of the mucous membrane of the nose and throat, can be transported by active infected carriers, and probably by healthy passive carriers of the specific micro-organism or virus, and fortunately is limited in its extension by a high natural indisposition or insusceptibility to infection existing among persons of all ages.

CHEMO-IMMUNOLOGICAL STUDIES ON LOCALIZED INFECTIONS.

FOURTH PAPER: EXPERIMENTAL PNEUMOCOCCIC MENINGITIS AND ITS SPECIFIC TREATMENT.*

By RICHARD V. LAMAR, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

The purposes of this paper are to record the general progress of the chemo-immunological studies, and in particular to show that a fatal experimental disease similar to a not uncommon and grave disease of man has often terminated in recovery after a treatment formulated from these studies, and to indicate a way in which the corresponding disease of man may be treated with a reasonable hope of sometimes preventing its almost invariably fatal termination.

In the first paper¹ has been related why and how we were led by consideration and experiment to attempt to arrest the progress of experimental pneumococcic infections by the local use of sodium oleate combined with immune antipneumococcic serum and boric acid. Among others, certain experiments were reported illustrating the results obtained by treating rats and mice inoculated in the peritoneal cavity. It was shown that appropriate treatment early after inoculation with massive doses of virulent pneumococci not only regularly saved the animals but also prevented the appearance of more than the slightest transitory signs of any illness. As a measure for preventing the development of infection the treatment was effective. As a curative measure for arresting the developing, or already established infection it was less effective, particularly in respect to the lapse of time after inoculation. Yet if we consider the severity of the test, the animals being highly susceptible to

* Received for publication, August 1, 1912.

¹ Lamar, R. V., *Jour. Exper. Med.*, 1911, xiii, 1.

rapidly fatal septicemia, the results were encouraging enough to lead us to pursue the principle in the hope of developing it, and because of the promise which it afforded of a possible successful application to the treatment of certain localized pneumococcic infections of man.

Since the publication of the first paper the work has been continued along the same fundamental lines in the search for an improved means of treatment. At the same time, pending developments in this direction, we undertook to ascertain whether what was already in hand might be successfully applied to the treatment of an experimental disease which bears a closer resemblance to pneumococcic diseases of man than is afforded by the septicemic infections of the small laboratory animals, and thus offers more reliable indications of the practical value of the therapeutic principle.

In the second and third papers² we recorded the results of the search for a more efficient agent than sodium oleate. Several substances were found to be much more powerful in destroying the pneumococcus in the test-tube, but they were less efficient than sodium oleate in controlling infections of the animal body. Also the attempts to find an agent which is superior to boric acid in preventing the serum inhibition of the sodium oleate failed. Hence we turned to the determination of the optimum proportions of the components of the therapeutic mixture of sodium oleate, immune serum, and boric acid, which had already proved itself.

What is meant by the optimum composition of the mixture will be apparent upon a brief restatement of the essential points regarding the action of the components alone and combined. The destructive action upon the pneumococcus of sodium oleate solutions varies throughout a wide range of concentration in direct ratio to the degree of dissociation. The destructive action is prevented wholly or in part by blood serum according to quantitative conditions. Alone in the animal body sodium oleate has little or no apparent restraining influence upon pneumococcic infections. In the test-tube a potent immune serum kills many of the pneumococci present but allows a subsequent rich growth of those which escape destruction, unless indeed a very small number be present, when all may be killed. The bactericidal action of immune serum does not depend

²Lamar, R. V., *Jour. Exper. Med.*, 1911, xiii, 380; xiv, 256.

wholly upon, but is enhanced by, the presence and coöperation of phagocytes; and the intensity of action varies in direct ratio to the degree of concentration of the serum. In the animal body immune serum exerts a distinct protective action, but one efficient in saving the animal only under quite sharply defined conditions pertaining to quantity of culture and of serum. In other words, there is a minimum quantity of serum which will protect against even the most minute, otherwise certainly fatal quantities of culture, and no quantity of serum protects against more than a definite quantity of culture. Dochez³ directed attention to this phase of the protective action of immune serum and has recently made it the subject of a special investigation. He has pointed out the relationship between quantity of serum and of culture in determining protection or lack of it, and suggested the importance of its bearing upon the practical treatment of established infections with immune serum. We attach an additional importance to the facts as affording a standard of comparison for appreciating the greater efficiency of the mixture of sodium oleate, immune serum, and boric acid. In contradistinction to test-tube experiments, a given quantity of serum in the animal body is just as efficient, if previously diluted even to 10 per cent. with salt solution, as if employed undiluted. The presence of boric acid in a certain quantity is necessary to prevent serum inhibition of the oleate, but an excess defeats the purpose of its employment, since of itself, in large quantities, it prevents the bacteriolytic action of the sodium oleate.

After testing several species of animals in diverse portions of the body in which we attempted to produce a suitable disease, with strains of the pneumococcus varying in virulence, we finally employed for our study pneumococcic meningitis in monkeys produced by a highly virulent pneumococcus. The conditions which this experimental disease presents are favorable for testing the method and important because of their close similarity to the highly fatal pneumococcic meningitis in man.

The experimental disease could be produced with ease, and, as it appeared later, with certainty. Except necessarily in the mode of infection it was remarkably like pneumococcic meningitis of man.

³ Dochez, A. R., Personal communication.

The clinical, bacteriological (including bacteremia), and pathological manifestations were nearly identical. It differed in that its course was more severe, and when untreated it terminated invariably in death. This statement is true of the disease produced, as it always was except once, by virulent pneumococcus. In a single instance a less virulent pneumococcus was injected and a meningitis produced that terminated in recovery without any intervention other than lumbar puncture performed for diagnostic purposes. The experiment has no bearing upon the particular theme of the paper, but is recorded to show how much the clinical result depends upon the infecting bacterium. It is an experimental confirmation of what clinicians are coming to realize, namely, that pneumococcic meningitis though a highly mortal disease is not necessarily always fatal; and it serves to indicate still further the close similarity of the experimental to the spontaneous disease.

The disease lent itself well, moreover, to constant observation of its course, and to direct local treatment, as lumbar puncture afforded indications of the influence of treatment during the course of the disease.

Before recording the individual experiments certain general features should be considered relating to the animal infected, the kind of pneumococcus employed, the mode of infection, and the method of treatment.

Macacus rhesus was chiefly used, but a few experiments were made with *Cercopithecus callitrichus*. All the monkeys were vigorous and seemed in good health. Genus, species, and sex appeared not to influence the results. But the individual factor was manifest. Most animals reacted to the inoculation in such a manner as to permit ready recognition of the type of the disease. Occasionally an individual proved excessively susceptible, seemed to oppose no resistance, and died of septicemia within the day. The monkeys seemed to be less resistant in the cold than in the warmer months. When and in what way these two factors of the individual and the season appeared to influence the experiment will be indicated in the discussion of the protocols.

The first three experiments were made with different strains of pneumococcus. They showed that a meningitis could be readily

produced, and allowed a study of the characteristics of the disease and the influence of degree of virulence of the bacterium upon its course. From the third experiment on, the same strain of pneumococcus was used throughout. This organism was obtained in pure culture by Dr. Dochez directly from the circulating blood of a patient with lobar pneumonia. Suffice it to say that it was a characteristic pneumococcus soluble in bile. In the beginning the organism was of high but not extraordinary virulence. It was first inoculated into monkeys after five passages in mice when 0.000,001 of a cubic centimeter was fatal. Repeated control tests showed the degree of virulence to be maintained throughout the ten months of study. In the nature of its pathogenic action the organism was well adapted to the purposes: it caused inflammation rather than a rapidly fatal septicemia.

In the first experiment the culture used was a virulent pneumococcus grown from the spinal fluid of a patient with meningitis. In the second one a so called avirulent pneumococcus isolated directly from the saliva of a well person was employed. The characteristics of this organism are stated in the protocol of monkey 2.

At the beginning a few experiments were made by intracranial inoculation. The disease produced in this way was most severe and quickly caused the death of the animal. Besides, it did not lend itself well to the treatment which is preëminently a local one administered by lumbar puncture. The opportunities for fluid injected intraspinally to reach the distant seat of severe inflammation in the brain itself were small. Normally in the monkey the space in the membranes is small and this is quickly further reduced by swelling and the fibrinous exudate. Indeed the focus of infection in the brain and its membranes seemed sometimes to have been cut off from the spinal subdural space. On account of the fibrinous nature of the pneumococcic inflammation this meningitic disease offers greater difficulties in treatment than, for instance, epidemic cerebrospinal meningitis. We shall refer later to those cases of pneumococcic meningitis which arise from middle ear disease and where the cerebral meninges are first and most inflamed.

Hence the intracranial method of infecting the animal was abandoned early, and throughout the remainder of the work the animals

were inoculated and treated by subdural spinal puncture and injection.

Two kinds of immune serum which were efficient in protecting rats and mice against infection by the same strain of pneumococcus used to produce the meningitis were tested alone. The one was from a goat, the other from a horse, immunized by the intravenous injection of large quantities of live, highly virulent, homologous pneumococci. Later other monkeys were treated with a mixture of sodium oleate, the same immune serum, and boric acid.

The experiments are presented in three groups. Those in the first group show the nature of the untreated disease and at the same time serve as control observations from which the results of treatment may be deduced. Those in the second group show the results of treatment with immune serum; and those in the third, treatment with the mixture of sodium oleate, immune serum, and boric acid.

THE EXPERIMENTAL DISEASE.

The first experiment was performed by Dr. Flexner in the spring of 1910. We abstract from his notes:

May 24, 1910. Monkey 1. *Macacus rhesus*; medium size. 2:00 P.M. Lumbar puncture; clear fluid; injected 0.4 c.c. of a thin suspension of a 24 hour culture on sheep serum agar, and a few drops of spinal exudate, from a case of pneumococcic meningitis (Schirck). 7:00 P.M. On perch; hair erected; not very sick. May 25, 10:00 A.M. Not very sick. L. p.;* small quantity of clear fluid containing a few polymorphonuclear leucocytes; no cocci. Injected 0.5 c.c. of a bouillon suspension from a fresh culture of same organism. 2:00 P.M. Monkey sick, lying partly down on perch. 4:00 P.M. L. p.; no fluid runs from needle. A little pus mixed with water remaining in needle is drawn up by the syringe. Film shows polymorphonuclear leucocytes and some Gram positive diplococci intra- and extracellular. May 26, 9:00 A.M. Animal lying on side; very sick. 10:00 A.M. General convulsion. 11:00 A.M. L. p. Cloudy fluid containing flakes of cells. Many leucocytes and diplococci some of which are intracellular. 11:10 A.M. General convulsion; some retraction of head; nystagmus; body limp.

May 27, died at 9:30 A.M. Autopsy at 11:00 A.M. The vessels of the cerebral meninges are injected and there is cloudy fluid in the meshes of the pia arachnoid, especially over the medulla. The ventricles contain an excess of cloudy fluid, but no purulent exudation. No macroscopic changes in the other organs. Films from the cerebral meninges and choroid plexus show many

* Abbreviation for lumbar puncture.

diplococci, nearly all extracellular. Many diplococci in heart's blood and spleen. Pure cultures of pneumococcus from surface of brain, lateral ventricle, heart's blood, and spleen.

The experiment requires little comment. Clinically there were many of those manifestations of meningitis which occur in man. Septicemia supervened. Death occurred early. At autopsy the familiar morphological and bacteriological conditions were encountered.

In the second experiment an organism of that variety which is commonly called avirulent was used. It was isolated directly from the saliva of a well person on January 25, 1911. The colony was of the characteristic ring form. In subcultures the organism exhibited the typical cultural and morphological characters of the pneumococcus; it fermented inulin feebly, and was soluble in bile. 0.5 of a cubic centimeter of a broth culture proved harmless to a mouse. From the time of its isolation until the date of this experiment, i. e., for ten weeks, the organism had been kept continuously on plain agar at 37° C. by third day transplantations.

Apr. 4, 1911. Monkey 2. *Cercopithecus callitrichus*; rather large. 3:30 P.M. Under ether anesthesia injected into cranium through fine trephine opening in left frontal region 1 c.c. of a 24 hour plain broth culture of pneumococcus L. 9. Animal conscious in five minutes. 4:30 P.M. Bright and active just as before inoculation. Apr. 5, 9:30 A.M. Animal sits on perch with head bowed; hair over anterior part of body erected. No paralysis or other focal symptoms. 3:30 P.M. A little worse; entire coat erect; little resistance to being captured. L. p. 1 c.c. of distinctly turbid fluid drops from needle; no coagula. Many pus cells and typical diplococci mainly extracellular; very little phagocytosis. Transplantation of one loop of fluid affords many colonies of pure pneumococcus. Apr. 6, 9:00 A.M. Animal lively; does not appear ill; coat smooth. 10:00 A.M. L. p. 4 drops of turbid fluid slowly. Many p. n. l.,^{*} but fewer than yesterday; moderate number of large, and a few small, mononuclear leucocytes. Fewer diplococci than in yesterday's fluid; no phagocytosis. Slight pure growth of pneumococcus. Apr. 7. Animal seems well. Apr. 10. Well. May 8. Continued well.

The experiment has already been referred to in the introduction. It led to the trial of another strain of pneumococcus, and one known to be virulent. The value of lumbar puncture in affording important information is apparent.

* Abbreviation for polymorphonuclear leucocytes.

In all the experiments now to be reported the virulent culture described before was used.

Apr. 9, 1910. Monkey 3. *Cercopithecus callitrichus*; very large. 11:30 A.M. Under ether anesthesia injected into cranium 1 c.c. of a 24 hour plain broth culture of pneumococcus X.5⁵*. Animal is recovering while the wound is being closed. Recovery complete in a few minutes. Apr. 10. Animal found dead at 7:00 A.M. Death occurred about 19 hours after inoculation.

9:30 A.M. *Autopsy*.—Upper lip retracted; both eyelids swollen; pupils one half dilated, round, equal. Lumbar puncture; two drops of slightly turbid fluid; very few p. n. l.; myriad diplococci; many typical pairs, also short chains; many degenerative forms. The brain shows yellow through the dura mater. The pia is milky and covered everywhere with a viscid exudate, greatest at the base of the cerebrum. The lateral ventricles contain a small quantity of the same exudate. The pia of the cord is milky everywhere, and there is a scant exudate. The ventral cavity of the body presents no gross changes. Films from the surface of the cerebrum, the lateral ventricle, and the spinal cord disclose many p. n. l. and a great many cocci in pairs and short chains, with only an occasional example of phagocytosis. In the heart's blood are many typical pairs and short chains. The spinal fluid, lateral ventricle, and the heart's blood all afford a pure profuse growth of pneumococcus.

The quantity of culture was large, and the disease fulminant, septicemia quickly supervening. The experiment indicates how quickly the spinal meninges may become involved and how rich the early exudate may be in fibrin. The bearing of the nature of the exudate upon the efficacy of treatment is most important, as will appear in the succeeding protocols.

The next experiment was performed in the same way with a smaller quantity of culture.

Apr. 10, 1911. Monkey 4. *Cercopithecus callitrichus*; medium size. 11:40 A.M. Ether anesthesia; injected intracranially 0.1 c.c. (1 c.c. of a 1 to 10 broth dilution) of a 24 hour broth culture of pneumococcus X.5⁵. Complete recovery in a few minutes. 5:00 P.M. Animal seems well. Apr. 11, 9:15 A.M. Animal sits bent forward slightly; coat slightly raised; eyes watery; pupils half dilated, round, and equal; respiration moderately accelerated. L. p. 3 drops of slightly turbid fluid very slowly; very few p. n. l.; few typical pairs of extra-cellular pneumococci. Moderate pure growth from one loop of fluid. P.M. Animal perhaps slightly worse. Apr. 12, 9:30 A.M. Worse; reclines on floor of cage in the corner; coat erect. Several attempts to obtain spinal fluid are unsuccessful. In the afternoon the animal's condition grows worse. Apr. 13,

* The Roman numeral refers to the name of the culture; the Arabic decimal indicates the serial number of the last mouse "passage"; and the exponent the generation in broth used.

A.M. Much weaker; lies on floor with head retracted. Made two unsuccessful attempts to obtain spinal fluid. During the afternoon the weakness slowly progresses.

Apr. 14. Found dead at 7:50 A.M. The body is fresh. Death occurred about 3½ days after inoculation. Autopsy 12 M. The inflammation is intense over the vertex of the cerebrum, and gradually diminishes towards the base and the medulla to become slight throughout the meninges of the cord. The cerebral cortex is yellow and the sulci are distended with pus and fibrin. The ventricles contain a moderate quantity of turbid fluid. Films from the brain show many p. n. l. and very many cocci in pairs and short chains; fewer in the scant spinal exudate. Profuse cultures from cortex, lateral ventricle, spinal cord, and heart's blood.

With the smaller quantity of injected culture the disease was slower in developing and the local inflammatory reaction was more marked. Spinal fluid could not be obtained after the end of the second day probably because of the thickening of the meninges and the absorption of the fluid by the fibrin in the exudate. The usual richness of the exudate in fibrin, a characteristic of pneumococcic inflammations in general, has in this particular disease an indirect effect in materially lessening the quantity of fluid which may be injected and in hindering its diffusion throughout the spinal canal and into the cranium.

The remainder of the experiments grouped under this heading were performed at intervals during the work largely for the purpose of often controlling the virulence of the culture, and the effect of treatment in companion experiments. In each of them the culture was injected by the spinal route.

May 8, 1911. Monkey 17. *Macacus rhesus*; medium size.

9:45 A.M. Injected, intraspinally, after the free withdrawal of 1.5 c.c. of clear fluid, 0.1 c.c. (1 c.c. of a 1 to 10 broth dilution) of a 24 hour broth culture of pneumococcus X.5^u. No symptoms. May 9, A.M. No signs of illness. 2:45 P.M. Animal is restless and eats less than usual. L. p. 1.2 c.c. of slightly turbid fluid freely. Sediment obtained by centrifugalization; many p. n. l.; few large and small mononuclear leucocytes; few extracellular diplococci. Profuse growth in culture. May 10, 9:00 A.M. Animal sits bowed on perch; hair raised slightly; weakness in movements upon being disturbed. 10:45 A.M. L. p. 12 c.c. less turbid fluid freely; several small white flakes; fewer cells; many cocci in pairs and a few short chains; slight phagocytosis. Profuse growth in culture. P.M. Animal becomes worse with great rapidity and dies at 5:00 P.M., 2½ days after inoculation.

Autopsy.—May 11, 9:00 P.M. The spinal membranes are moderately congested, moist, and the pia is milky. No fibrin is visible. The cerebral meninges

are glued together, and the cortex tears in several places as the dura is being reflected. There is a general moderate congestion most marked at the base. The pia is cloudy everywhere and opaque in the sulci. The ventricles contain a small quantity of rather thick yellowish fluid. In films from the cortex there are a great many pus cells and many diplococci for the most part extracellular and only fairly well preserved. There is moderate phagocytosis. In the spinal exudate there are fewer pus cells and diplococci and less phagocytosis. On the surfaces of the lower lobes of both lungs is much mucilaginous exudate in sheets and clumps containing many poorly staining pus cells and a moderate number of typical pneumococci, of which a few are intracellular. Otherwise the ventral cavity is negative. No bacteria seen in the heart's blood; culture negative. From the cerebral and spinal exudate the pneumococcus grows profusely and pure.

At first the animal offered considerable resistance, shown by its physical condition, the polymorphonuclear cell reaction in the spinal fluid, and the slow multiplication of the diplococcus. Then it showed signs of illness and became worse with great rapidity, while coincidentally the cellular reaction subsided and the cocci grew profusely. The absence of cocci from the blood at autopsy indicates that the meningitis itself may cause death without the supervention and help of septicemia. There was a metastatic pleurisy of slight extent and degree.

The experiment reported next was done in the autumn after the summer interruption of two months, during which time the culture was preserved in the dried spleens of infected mice.

Sept. 19, 1911. Monkey 32. *Macacus rhesus*; medium size.

11:00 A.M. L. p.: three attempts to obtain fluid failed; on the third attempt, injected 0.1 c.c. (1 c.c. of a 1 to 10 broth dilution) of a 24 hour broth culture of X.g¹. With the needle and syringe still in place withdrew and reinjected 0.3 c.c. for assurance. No symptoms. During remainder of the day the animal seems well. Sept. 20, 9:00 A.M. Animal not so active as yesterday. 11:00 A.M. Ill; rather quiet; deliberate in movements; eyes dull; coat beginning to rise. 2:00 P.M. All symptoms more marked; weakness evident as animal springs; head retracted at times. 4:00 P.M. Worse; coat much raised. Sept. 21, A.M. Animal is worse; quite weak, but still moves about; coat rough. P.M. Condition about the same. Sept. 22, 9:22 A.M. Animal much worse; no longer jumps but climbs slowly up grating of cage to perch. P.M. Very weak; sits quietly huddled; does not move when touched. Sept. 23, 9:00 A.M. Prone; apparently dying; head much retracted. Death at 10:30 A.M., 4 days after inoculation.

Autopsy.—Performed at once. The brain and cord are moist. The pia everywhere is thickened, opaque, and yellowish white, more over the cerebral hemispheres than along the cord. The ventricles are slightly distended with turbid

fluid. Diplococci are present all along the cord and over the brain beneath the dura. They are not numerous except in the fibrinous exudate covering and infiltrating the pia of the convexity of the brain, where there are enormous numbers. Phagocytosis is slight. At the bases of both lungs there are a few deposits of a dry fibrinous exudate containing pus cells and diplococci. From the cortex, lateral ventricle, spinal cord, and pleural exudate the pneumococcus grows profusely; from the heart's blood, in a few isolated colonies.

The experiment indicated that the organism had preserved its virulence. The disease progressed gradually and steadily. Again the inflammation appeared to be greater in the cerebral than in the spinal meninges although the inoculation was made into the spinal canal. The dry pleurisy occurred again. And, as in the preceding experiment, septicemia did not seem to be necessary to cause death. The few cocci found in the blood at autopsy were probably the expression of an agonal invasion rather than of a true septicemia. The animal of this experiment was the companion to one inoculated at the same time in the same way and treated later (compare monkey 33 of the third group).

Dec. 12, 1911. Monkey 45. *Macacus rhesus*; medium size. 1:55 P.M. L. p.: about 0.7 c.c. of clear fluid; injected 0.1 c.c. of a 22 hour broth culture of X.11^r. Slight retraction of back upon completion of injection passing away in a few seconds. For the remainder of the day until 6 o'clock, when it is observed for the last time, the animal seems perfectly well. Dec. 13. Animal found dead at 8:00 A.M. Lived about 15 or 16 hours after inoculation.

Autopsy.—At 9:30 A.M. Body fresh. There is a moderate quantity of yellow, sticky exudate all along the spinal pia, and over the brain, being most abundant at the base. The ventricles contain a modicum of opalescent fluid. In the meningeal exudate everywhere there are many pus cells and enormous numbers of diplococci, many of which are intracellular. Cultures are profuse and pure. Films from the left lateral ventricle show a few mononuclear cells, no p. n. l., and no cocci. No growth upon transplantation from this source. Heart's blood: moderate number of typical capsulated pairs and a few short chains; profuse growth in culture. Both lungs are slightly congested and edematous, but there is no consolidation. Otherwise the ventral cavity is negative.

This experiment shows the fulminant disease. It is remarkable that so intense a purulent inflammation with fibrin formation could occur in such a short time. At this season, during the cold of mid-winter, the disease ran regularly a shorter course than it had done in the spring and autumn. Possibly also this animal was unduly susceptible, for no change was ever made in the manner of preserv-

ing and of using the culture, which in all other tests exhibited what is commonly accepted as maximum virulence.

Two more experiments to illustrate the untreated disease very similar to the one just described may be summarized. The monkeys were inoculated in the usual way late in the afternoon of December 14, 1911, and on January 5, 1912, respectively. Seen twenty and forty-five minutes later the animals seemed well; but both died during the night, one about thirteen hours, and the other about nineteen hours after the inoculation. The autopsy in each instance showed a general purulent cerebrospinal meningitis and septicemia. The rapidity of the development is evident.

It was, as already stated, in mid-winter that the disease was so much more severe than it had been before. Therefore the quantity of culture inoculated was reduced to one fifth of what had been regularly used before. The protocols of the experiments appear in the second and third groups.

To summarize: the experiments show that virulent pneumococci when injected into the posterior cavity of monkeys produce regularly an inflammation of the meninges, and particularly of the pia mater, attended by bacteremia; that the inflammation becomes quickly purulent and fibrinous in character, extends readily from the cerebral to the spinal meninges and *vice versa*, and is so regularly attended by certain definite general symptoms that the whole constitutes a definite disease entity.

INFLUENCE OF TREATMENT WITH IMMUNE SERUM.

In all, eleven experiments were made with immune serum. In two not only the animal was treated but also the culture before it was inoculated. That is, in order to make the simplest test of the serum, a large quantity, many times that required to protect a rat against the same quantity of culture as the monkeys received, was added to the culture, the mixture incubated, and then inoculated into the monkey, which later received still more serum. In nine experiments of this group, as well as in all those of the third group, the animal was first inoculated with the culture alone and then later treated. The time allowed to elapse between inoculation and

the beginning of treatment was usually determined by the physical condition of the animal and the character of the spinal fluid. As a rule, in the treatment with immune serum alone, this interval was short. The results of early treatment were so little encouraging that there was no reason to increase the interval between inoculation and the beginning of serum treatment. Once begun the treatment was usually repeated once a day as long as the animal lived; or, as occurred in two instances, until the physical condition of the animal was much improved and the spinal fluid gave no growth of the coccus. The quantity of serum injected was the maximum of what was considered safe, and was determined by the quantity of fluid which had been withdrawn, and the condition of the animal while the slow injection was being made. In the beginning of the disease it was almost always possible to inject two cubic centimeters with no apparent embarrassment to the animal; later, as the disease progressed, less spinal fluid could be withdrawn and less serum injected, but even then it was usually possible to give at least one cubic centimeter without appreciable harm. In some experiments concentrated serum was used; in others a 40 per cent. dilution. Of itself the serum seemed to be harmless.

Apr. 14, 1911. Monkey 9. *Cercopithecus callitrichus*; large size.

9:55 A.M. Under ether anesthesia injected intracranially 0.1 c.c. of a 24 hour broth culture of X.5⁴. Recovery complete in a few minutes. 1:55 P.M. No signs of illness noticed. L. p.: 0.5 c.c. of slightly blood-tinged fluid. In the sediment there are no more leucocytes than the presence of blood accounts for, and no cocci are seen. Twelve colonies of pneumococcus grow from the planted fluid.

Injected 2 c.c. of immune serum (goat 5, bleeding of February 16, 1911). No symptoms attend or follow injection.

Apr. 15, A.M. Animal is slightly ill; sits quietly but moves with agility when disturbed; coat somewhat fluffy. 10:15 A.M. L. p.: 2 drops of clear fluid; very few cells, about half of which are polymorphonuclear and half mononuclear; no cocci seen. Scant growth of pneumococcus in culture. Injected 2 c.c. of the same immune serum.

Apr. 16, 10:15 A.M. Animal slightly worse; not so quick in movements; head droops a little. L. p.: no fluid obtained. Injected 2 c.c. of immune serum without resistance. Apr. 17, 10:30 A.M. Slightly worse. L. p.: no fluid obtained. Injected 2 c.c. of immune serum; slight struggle towards close of injection. Until noon the animal remains in about the same condition. P.M. Much worse; lies down sometimes; no longer eats.

Apr. 18, 10:00 A.M. Very weak; lies on side, eyes swollen. L. p.; no fluid

obtained; believing that the animal would die if left alone, we injected again 2 c.c. of immune serum. The last half of the injection is attended with slight struggle and embarrassment of respiration. During the remainder of the day the animal's condition seems about the same as before the injection. Apr. 19. Animal found dead at 8:00 A.M. It died 4½ days after inoculation.

Autopsy at 10:00 A.M. Intense inflammation of cranial meninges; the sulci are widely distended by a purulent exudate rich in fibrin. There is a small area of red softening on the superior surface of the right lobe of the cerebellum. The spinal cord is enveloped throughout by a yellowish sheath about 2 mm. thick representing the pia and the fibrinopurulent exudate. There are many p. n. l. and enormous numbers of cocci in pairs and short chains in the cerebral and spinal exudate. Phagocytosis is slight. In culture profuse pure growth of pneumococcus from exudate and heart's blood.

In this experiment treatment was begun early and only four hours after the inoculation while the animal still seemed well; and although the bacteria had already reached the spinal fluid probably only slight multiplication had occurred, for they were too few in number to be seen in films. Even on the second day the animal was still very strong. At the best the serum only delayed the fatal issue a little while. It may not have reached the brain in more than small quantities much diluted, but even in the spinal canal where it was present and could not have been greatly diluted it did not prevent the growth of the cocci and the production of an inflammation almost as severe as that in the cerebral meninges.

Apr. 21, 1911. Monkey 11. *Macacus rhesus*; large.

11:10 A.M. Under ether anesthesia injected intracranially 0.1 c.c. of a 24 hour broth culture of X.5⁶. Complete recovery at once. 3:10 P.M. Animal seems a little torpid; otherwise no signs of illness are detected. L. p.: 1.2 c.c. of turbid fluid freely; moderate number of p. n. l., more than a moderate number of extracellular diplococci. Profuse growth in culture. Injected easily 2 c.c. of immune goat serum. No symptoms. Also injected intravenously 5 c.c. of immune serum. This injection is followed by a mild shock of short duration. Seven minutes later the animal has recovered completely. 4:30 P.M. The animal huddles against another in the cage; moves with agility when disturbed; coat slightly raised. The animal was observed no more this day, and was found dead at 7:30 the next morning. It probably lived 8 or 9 hours after the inoculation.

Autopsy.—11:30 A.M. The membranes of the brain, chiefly over the convexity, are milky from the presence of a moderate quantity of yellowish white exudate containing some fibrin. Films show many p. n. l. and very many diplococci. In the spinal canal there is less exudate but yet p. n. l. and many diplococci. The heart's blood contains many diplococci. Profuse growth of pneumococcus occurs in all cultures.

In this experiment the animal was unusually susceptible, for in four hours it had become ill and the bacteria had reached the spinal fluid and begun to multiply. It was on this account that the intravenous injection of immune serum was made. At least it was not effective in delaying death. The experiments were so little encouraging that an attempt was made to afford the serum better opportunities to exert any beneficial action of which it might be capable. To this end the culture and serum were mixed and incubated and then injected in order to see if infection would be prevented.

Apr. 21, 1911. Monkey 12. *Macacus rhesus*; small.

4:00 P.M. Under ether anesthesia injected intracranially a mixture consisting of 0.1 c.c. of a 24 hour broth culture of X.5^a and 1 c.c. of immune goat serum. The mixture had been incubated for 1 hour and 10 minutes at 37° C. and then kept in the refrigerator for 30 minutes. A culture of the mixture just before it was injected furnished a moderate growth of pneumococcus. The animal quickly recovered and seemed well for the remainder of the day. Apr. 22. The animal may be a little agitated; otherwise it seems well. 11:00 A.M. L. p.: 1 c.c. of turbid fluid freely; a moderate number of p. n. l., a few mononuclear cells, and no cocci. No growth in culture. Apr. 23. Animal seems well throughout the day.

Apr. 24, A.M. Still seems well. At 3:30 P.M. the animal is first noticed to be ill. It is greatly agitated, shaking at the grating of the cage. In a few minutes it lies prone and trembling with roughened coat. On being taken up an almost complete flaccid paralysis of the left anterior extremity and a spastic paresis of the left posterior extremity is apparent. There is slight ptosis of the left upper eyelid. 4:00 P.M. Animal weaker. L. p.: turbid fluid flows quickly under pressure; a few p. n. l. and mononuclear cells; more than a few diplococci; no phagocytosis; profuse growth in culture. Injected immune goat serum, stopping at 1.6 c.c. upon the appearance of struggle and slight embarrassment of respiration; recovery in one minute. 6:00 P.M. Condition about the same as before the injection. Animal found dead at 7:30 P.M., 3½ days after the inoculation.

Autopsy.—April 25, 10:30 A.M. There is an intense fibrinopurulent inflammation of the pia of the brain and cord, which is more pronounced in the brain where the sulci are widely distended by a thick yellow exudate. The cortex everywhere is much congested, even within its substance. There is a large area of red softening in the right temporal lobe. The ventricles contain a moderate quantity of turbid fluid. In films from the brain and cord there are many p. n. l. and enormous numbers of diplococci everywhere. Profuse growth in cultures from the brain and heart's blood.

In this experiment the immune serum had already effected a considerable reduction in the number of viable cocci in the culture be-

fore it was injected. The condition of the animal for the first two days and the character of the spinal fluid seemed to indicate that the infection if not prevented was retarded. Then symptoms of illness appeared more or less suddenly and the animal very rapidly weakened and died at about the time the untreated controls did. The autopsy indicated that the disease was developing all the while the animal showed few or no chemical signs and explained the focal symptoms.

It was at this point that the intracranial method of inoculating was abandoned and the intraspinal substituted. The next experiment resembled the one just described except that the mixture of culture and serum was injected into the spinal canal, and the animal itself was later given a regular course of serum treatment regardless of its apparent condition.

Apr. 25, 1911. Monkey 13. *Macacus rhesus*; medium size.

1:30 P. M. Injected intraspinally a mixture consisting of 0.1 c.c. of a 24 hour broth culture of X.5⁶ and 1 c.c. of immune goat serum. The mixture had been incubated for one hour at 37° C. A transplantation from it just before it was injected furnished a moderate growth of pneumococcus. No symptoms followed the injection. Remainder of the day animal seems well. Apr. 26. Animal seems perfectly well. 10:00 A.M. L. p.: 1.5 c.c. of moderately turbid fluid freely; a moderate number of p. n. l. and a few mononuclear cells are seen in films. There are a few bodies, about half intra- and half extracellular, which appear to be swollen cocci. Here and there an occasional diplococcus is identified. Transplantation gives rise to no growth. Injected 1.8 c.c. of immune serum; considerable struggling. During the afternoon the animal seems well. Apr. 27, 2:50 A.M. No evidence of illness. L. p.: 1 c.c. of fluid, about half as turbid as that obtained yesterday, flows slowly; p. n. l. predominate; no bacteria seen; no growth occurs. Injected 2 c.c. of immune serum; only slight struggling. Apr. 28. Animal seems perfectly well. 9:45 A.M. L. p.: 1 c.c. of faintly turbid fluid; a few cells about half of which are mononuclear; no bacteria seen; no growth occurs. Injected 1.8 c.c. of immune serum; much struggling. Apr. 29. Well.

The animal remained well for one month when it developed diarrhea, became emaciated rapidly, and died on June 30.

Autopsy at 4:00 P.M. Body much emaciated. Nothing abnormal is discovered in the central nervous system. There is an extensive ulcerative enteritis and hyperplasia of the mesenteric lymph nodes. Transplantations from the cerebral cortex, lateral ventricle, spinal cord, and heart's blood give rise to no growth.

In this experiment the immune serum was given the maximal advantage. When incubated with the culture before inoculation

and later administered three times to the animal it prevented the occurrence of infection. Such prevention of infection, while undoubtedly indicative of a beneficial action of the serum, is to be sharply distinguished from the arrest of an established infection. In the next experiment the test of the serum was made somewhat more difficult.

May 1, 1911. Monkey 16. *Macacus rhesus*; medium size.

11:50 A.M. Injected intraspinally 0.1 c.c. of a 24 hour broth culture of X.5⁺. At 1:50 P.M. animal well. L. p.: 3 drops of opalescent fluid slowly; a few mononuclear cells and an occasional pair of cocci in films; slight growth in culture. Injected 2 c.c. of immune goat serum. Animal seems well remainder of the day. May 2, 9:40 A.M. Animal may be a little agitated; otherwise no signs of illness can be detected. L. p.: about 0.7 c.c. of a slightly turbid fluid containing one small white flake; a fair number of white cells, half p. n. l. and half mononuclear; no bacteria seen; no growth. Injected 2 c.c. of immune serum. May 3, A.M. The animal eats with normal greed and seems well. 11:10 A.M. L. p.: 1 c.c. of opalescent fluid slowly; more white cells than yesterday; ratio about the same; no bacteria seen; no growth. Injected 2 c.c. of immune serum. May 4, 10:05 A.M. Animal has continued well. L. p.: 2 drops of clear fluid; an occasional p. n. l. and a very few mononuclear cells; no bacteria seen; no growth. No serum injected.

The animal was kept under observation for two months during which time it remained well.

Immune goat serum given two hours after the inoculation and once on each of the two succeeding days seemed to prevent multiplication of the bacteria and the occurrence of infection, although the animal was apparently intoxicated for one day after the inoculation.

These two were the only instances in which an immune serum prevented the infection and death of the animal. In all other experiments where a longer period than two hours was allowed to elapse between inoculation and the beginning of treatment not only did all of the animals die, but the disease followed much the same course as in the untreated control animals. These experiments are summarized in table I (page 598).

The experiments call for little discussion. They show that immune serum had a distinct, though only slight, restraining influence upon infection. When administered within two hours it prevented the occurrence of infection in two instances. When given

TABLE I.

Date.	Designation of animal.	Time elapsing between inoculation and beginning of treatment.	At beginning of treatment.		Subsequent treatments.	Result.	Summary.
			Condition of animal.	Nature of spinal fluid.			
May 10, 1911	No. 19 ⁷	8 hrs.	Slightly ill, excited.	Very few p.n.l., many extracellular diplococci; profuse growth.	None.	Died 20 hrs. after inoculation.	Septicemia; animal was probably less resistant than the average.
May 12, 1911	No. 21 ⁷	4 hrs.	Slightly ill.	Slight multiplication of cocci; slight growth.	1 and 2 days later.	Died 2½ days after inoculation.	Serum seemed to exert some restraint on growth of cocci; no septicemia.
Oct. 10, 1911	No. 36 ^a	18 hrs.	Apparently well.	Few p.n.l.; myriad diplococci; profuse growth.	1 and 2 days later.	Died 3½ days after inoculation.	Bacteremia throughout illness; encephalitis; cerebral softening. Septicemia.
Jan. 11, 1912	No. 50 ^a	10 hrs.	Perhaps a little excited; otherwise well.	Very few diplococci, some intracellular; scant growth.	1 day later.	Died 5½ days after inoculation.	The 2d lumbar puncture revealed no cocci either in films or culture, and the animal seemed well on the 2d, 3d, and 4th days. On the morning of the 5th day, the animal showed signs of "relapse," weakened very rapidly, and died a few hours later.
Jan. 24, 1912	No. 52 ^a	8½ hrs.	No signs of illness.	Many p.n.l. and extracellular diplococci; profuse growth.	1 and 2 days later.	Died 2½ days after inoculation.	The first injection seemed to restrain growth of the cocci, the second and third did not do so appreciably.
Jan. 30, 1912	No. 53 ^a	8½ hrs.	Slightly ill; restless.	Many p.n.l. and extracellular diplococci; profuse growth.	None.	Died 21 hrs. after inoculation.	Inflammation mainly cerebral; no spinal fluid at autopsy. Many diplococci in cerebral exudate. No septicemia.

⁷ Immune goat serum was employed.^a Immune horse serum was employed.

later the first injection seemed usually to restrain infection, but the restraining action was of short duration, the disease quickly developing into its usual course and producing death. An even apparently beneficial action of subsequent injections was rare. Thus the serum was utterly powerless to stop an infection once well begun and to prevent the death of the animal.

INFLUENCE OF TREATMENT WITH A MIXTURE OF SODIUM
OLEATE, IMMUNE SERUM, AND BORIC ACID.

In the experiments of this group the animal was first inoculated in the spinal canal. Afterwards, according particularly to the animal's physical appearance and the state of the spinal fluid, treatment was begun. As a rule, the treatment was repeated once a day during the life of the animal, or until the spinal fluid gave no, or very little, growth of pneumococcus. Each cubic centimeter of the mixture contained 0.1 of a cubic centimeter of a 1 per cent. aqueous solution of Merck's or Kahlbaum's sodium oleate, 0.2 of a cubic centimeter of the immune antipneumococcic serum, and 0.7 of a cubic centimeter of a 5 per cent. aqueous solution of boric acid. The mixture was always freshly prepared for each injection. In the protocols it is designated by the letters T. M. As in the experiments with immune serum, usually two cubic centimeters were injected each time during the early stages of the experiment, later it was often not possible to inject more than one cubic centimeter without causing symptoms of pressure. Of itself the mixture was harmless, both in infected animals and in normal ones employed to determine this point.

The nineteen experiments will be first recorded in the form of a table (table II) showing the gross features and result of each, after which a few that possess features of special importance or interest will be described at greater length.

From the table it is apparent that in those instances in which recovery took place it did so only after repeated injections of the mixture of sodium oleate, immune serum, and boric acid. In only one instance, that of monkey 28, were as few as three treatments given. The average number was five or six. This means that an actual disease was treated, as was clear from the animal's general

physical condition, its behavior, and from the continuous presence of pus and live pneumococci in the cerebrospinal fluid. After each treatment it was usual to notice an improvement in the physical condition, a clearing of the spinal fluid, and a reduction in the number of diplococci. This was true particularly of the first injection, which was usually followed by a disappearance of the bacteria from the circulating blood, and not only in those instances where recovery occurred but also often even in those which terminated fatally. The subsiding of the bacteremia is probably due, as Dr. Flexner¹¹ has said in his lecture upon local specific treatment, not only to the control of the local infection but also to the action of immune principles which have diffused from the cerebrospinal fluid into the general circulation.

An example of the disease terminating in recovery is afforded by the protocol of monkey 35.

Sept. 26, 1911. Monkey 35. *Macacus rhesus*; fairly large.

4:05 P.M. L. p.: clear fluid; injected 0.1 c.c. of a 20 hour broth culture of X.11¹ (made directly from the heart's blood of a mouse). At 6:00 P.M. no change is apparent. Sept. 27, 9:00 A.M. Animal slightly ill; eyes watery and not so bright as yesterday; coat raised. 10:04 A.M., 18 hours after inoculation. L. p.: 2.5 c.c. of opalescent fluid quickly, under pressure. Films from the sediment show a few p. n. l. and about as many mononuclear cells; an occasional pair of extracellular diplococci. Culture gives moderate growth of pneumococcus.

Injected 2 c.c. of the mixture of sodium oleate, immune serum, and boric acid (T. M.) No symptoms. Throughout the remainder of the day the animal may be a little better.

Sept. 28, 9:00 A.M. Condition about the same; animal still moderately ill. 9:15 A.M. L. p.: 2 c.c. of turbid fluid freely; many p. n. l. and a few mononuclear cells; no cocci seen; no growth.

Injected 2 c.c. of T. M. Condition remains the same remainder of the day.

Sept. 29, 10:50 A.M. Animal better; coat still fluffy. L. p.: 1.3 c.c. of turbid fluid freely; a great many p. n. l. and many capsulated extracellular diplococci; an occasional pair intracellular; profuse growth. Injected 2 c.c. T. M. Sept. 30, 8:45 A.M. Condition same as yesterday. L. p.: 1 drop of turbid fluid; many p. n. l.; fewer diplococci, some involuted and Gram negative, and an occasional pair intracellular. Profuse growth but less than yesterday. Injected 1 c.c. of T. M. Oct. 1, A.M. Condition the same. 4:05 P. M. L. p.: 4 drops of slightly blood-stained fluid; few p. n. l., no cocci seen. Two colonies in the culture. Injected 2 c.c. of T. M. Oct. 2, A.M. Animal decidedly better; eats more and with some greed. P.M. Condition unchanged. Oct. 3. Better.

¹¹ Flexner, Simon, *Boston Med. and Surg. Jour.*, 1911, clxv, 709.

in the spinal fluid was found to have taken place; followed one injection of serum.

dense and profuse growth. severely

in the spinal fluid was found to have taken place; followed one injection of serum.

date	No.	condition	spinal fluid	post-mortem	notes
Nov. 6, 1911	No. 41 ¹⁰	45 hrs. Moderately ill; bacteremia for two days.	Many p.n.l.; a few extracellular diplococci; slight phagocytosis; profuse growth.	1, 3, 4, 5, 10, 11, 12, days later.	Died 14 days after inoculation. Irregular course. The intermission in the treatment corresponded to days of great improvement and failure to obtain cultures from the spinal fluid. Marked anemia and emaciation during the second week. Autopsy: cerebral meninges intensely inflamed, the spinal slightly.
Nov. 27, 1911	No. 43 ¹⁰	20 hrs. Extremely ill, and rapidly becoming worse; septicemia.	Enormous numbers of p.n.l.; myriad diplococci nearly all extracellular; very profuse growth.	None.	Inoculated in the evening and found moribund the next morning.
Dec. 5, 1911	No. 44 ¹⁰	20 hrs. Slightly ill; bacteremia.	Fluid coagulates; great many p.n.l.; very great many diplococci extracellular; profuse growth.	1, 2, 2½, 3, 4, 5, and 6 days later.	Much improvement after first treatment; then slow progress to complete recovery in ten days.
Dec. 14, 1911	No. 46 ¹⁰	16 hrs. Gravely ill; septicemia.	Many p.n.l.; myriad extracellular diplococci; very profuse growth.	2 and 5 hours later.	Great destruction of bacteria and marked general improvement followed the first treatment, but bacteria soon multiplied. Little restraint by the 2d and 3d treatments. Inflammation most intense in cranium. Septicemia.
Jan. 8, 1912	No. 49 ¹⁰	18 hrs. Very ill; sits motionless; cannot be roused; bacteremia.	Very many p.n.l.; myriad cocci in pairs and short chains; occasional phagocytosis.	4 hours and 1 day later.	The first treatment was followed by improvement which lasted a few hours. Septicemia; dry peritonitis.
Jan. 17, 1912	No. 51 ¹⁰	8½ hrs. Moderately ill; lies down at times.	Very many p.n.l.; few extracellular diplococci; profuse growth.	15 hours and 2, 3, 4, 6, and 12 days later.	Improved steadily after the first treatment. At the 6th treatment the spinal fluid was sterile. Animal well until the 12th day, when it became a little excited. L. p. gave clear sterile fluid, but a precautionary treatment was given. From the next day on animal perfectly well.

* The therapeutic mixture contained immune goat serum.

** The therapeutic mixture contained immune horse serum.

(LAMAR: Chemo-Immunological Studies on Localized Infections.)

Oct. 4. Still improving. Oct. 6. Animal well. Yet fearing a possible relapse (compare No. 34) l. p. was done at 4:00 P.M. 3 drops of blood-tinged fluid; no excess of p. n. l. over normal ratio to red blood cells; no cocci seen; two colonies in culture. Injected 1 c.c. of T. M.

Nov. 5. Observed daily, animal has shown no signs of illness until today when there is diarrhea. Nov. 7. Diarrhea worse; emaciation. Nov. 10. Animal found dead this morning. Autopsy at once: moderate emaciation. Nothing abnormal found in the posterior cavity. There is an acute colitis of severe degree; hyperplasia of the mesenteric lymph nodes.

Upon microscopic examination nothing abnormal is found in the spinal cord or its membranes.

In the experiments which terminated fatally the disease followed one of two well characterized and readily recognizable courses. Either the animal was gravely ill when the treatment was begun and died during the first or second day, nearly always from septicemia; or the animal was not so ill at the beginning of treatment, the customary improvement followed the first injections, recovery seemed to have taken place, the treatment was discontinued, and then later what may be designated a relapse occurred and was quickly followed by death. In one instance (protocol 39), after apparent recovery a massive fibrinous pneumonia, instead of a relapse, developed and led to death. The meningitis was healed.

The first type of fatal disease is illustrated by the protocol of monkey 43.

Nov. 27, 1911. Monkey 43. *Macacus rhesus*; medium size.

1:40 P.M. L. p.: clear fluid; injected 0.1 c.c. of a 24 hour broth culture of X.11⁸. No evidences of illness during remainder of day. Nov. 28, 8:00 A.M. Animal quite ill. 9:15 A.M. Very ill. 9:35 A.M. Seems worse than twenty minutes ago; very weak; can no longer sit up. L. p.: 1.3 c.c. of very turbid fluid containing flocculi; very many p. n. l. mostly poorly staining and an enormous number of diplococci nearly all extracellular. Very profuse growth. Likewise profuse growth from four drops of blood taken from the ear. Leucocytosis about 25,000. Injected 1.5 c.c. of T. M. No immediate change in the animal's condition, but it steadily weakens. Death at 12:15 P.M., 22½ hours after inoculation.

Autopsy.—1:30 P.M. Slight congestion of spinal meninges. There is a scant, sticky, yellowish white exudate all along the surface of the pia. It contains many p. n. l. and still a great many diplococci but not nearly so many as were in the fluid drawn off prior to the treatment. Extreme phagocytosis has occurred. Most cocci are within pus cells and many pus cells are crowded with the organisms. Cultures furnish moderate growth. The cerebral pia is more congested than the spinal, and there is more exudate. In the heart's blood are a few diplococci. Profuse growth occurs from it.

The second type of fatal disease, that of apparent recovery followed, upon discontinuation of treatment, by late relapse and death, is illustrated by the protocol of monkey 29.

May 25, 1911. Monkey 29. *Macacus rhesus*; medium size.

10:50 A.M. L. p.: 1 c.c. of clear fluid; injected 0.1 c.c. of a 24 hour broth culture of X.5th. 2:15 P.M. Animal uneasy. 5:30 P.M., 7 hours after inoculation. Animal slightly ill; hypersensitive along spine; coat ruffled. L. p.: 2 c.c. of turbid fluid; many p. n. l., an occasional pair of cocci extracellular; more than moderate growth. Injected 2 c.c. of T. M. May 26, 10:30 A. M. Somewhat better. L. p.: 2 c.c. of turbid fluid; more p. n. l. than yesterday; only one pair of diplococci seen; a single colony grows from the transplant. Injected 2 c.c. of T. M. Condition remains the same during remainder of day. May 27, A.M. Much better; animal springs up and down as normally. May 28. Well. May 29. Still seems well.

May 30, 9:20 A.M. The animal is found with coat slightly raised, sitting on its perch. It cannot be made to come down; cries when disturbed. Made many attempts, in several places, to obtain spinal fluid; all failed; even three made under light ether anesthesia. Finally, injected with little resistance 1.5 c.c. of T. M.; no pressure symptoms observed. The animal quickly recovers from the ether and upon being put into its cage climbs upon the perch and remains sitting as before. 2:00 P.M. Still sits upon perch; seems about the same. 4:30 P.M. Animal dead. Death occurred 5½ days after inoculation.

Autopsy.—May 31, 9:30 A.M. The spinal cord beneath the dura is enveloped throughout its length by a thick, yellowish white, translucent sheath comprising the pia infiltrated and covered by a fibrinous exudate, the whole averaging from 1 to 2 mm. in thickness. Films show many p. n. l. in all stages of disintegration, and an occasional coccus, but so few that only after a search of several minutes are a few swollen pairs found within a single pus cell.

The pia of both cerebral hemispheres and to a less extent that of the cerebellum is finely congested and studded with abundant punctate hemorrhages. Films from the cerebral cortex contain many well preserved p. n. l. and many typical extracellular diplococci. There is less similar exudate at the base of the brain. The ventral cavity of the body is negative. Cultures from the exudate in the lumbar and thoracic regions of the spinal cord and from the heart's blood remain sterile. From the right cerebral cortex the pneumococcus grows abundantly and pure.

In this experiment the effect of the injections in almost sterilizing the spinal canal is apparent. The clinical "relapse" and death of the animal were evidently due to the progress of the inflammation in the cranial cavity which had been cut off from communication with the spinal subdural space by the fibrinous exudate.

DISCUSSION.

The experimental data presented have more than a theoretical interest. They should indeed be considered with respect to their application to the treatment of a highly fatal disease of human beings. Pneumococcic meningitis is far from being a rare affection, and the number of reported recoveries from the disease is very small. There exists at present no effective treatment that has come to be at all generally employed. The few instances in which anti-pneumococcic serum has been applied have not yielded results that inspire confidence in its sole employment. Even when administered by direct intraspinal injection the effects have been doubtful. Matthes¹² so treated three patients and all died; Grober,¹³ two, one of which recovered and one died; and Schlesinger¹⁴ and Kleinschmidt,¹⁵ one each, both recovering. The total number is small, and although three of seven treated with serum recovered there have been reported ten more instances of recovery following no more radical treatment than mere lumbar puncture and withdrawal of fluid.¹⁶ The reasons for the inefficiency of serum treatment are now rendered fairly clear. An antipneumococcus serum is at best active only against the homologous organism or organisms the types of which have been employed in its preparation. Under optimal experimental conditions the extent of its efficacy is confined within a brief space of time; and in general this will probably be found equally true of the so called spontaneous human pneumococcus meningitis. The outlook for its successful employment alone in the human disease is not encouraging.

The therapeutic mixture of sodium oleate, boric acid, and anti-serum holds out greater promise. The scope of its efficacy is far wider than that of the antiserum alone. It remains still to be determined whether its action is as closely restricted within the limits of homologous strain of pneumococcus as is that of the anti-

¹² Matthes, M., *Med. Klin.*, 1908, iv, 733.

¹³ Grober, J., *München. med. Wchnschr.*, 1910, lvii, 1332.

¹⁴ Schlesinger, H., *Wien. med. Wchnschr.*, 1911, lxi, 40.

¹⁵ Kleinschmidt, *Med. Klin.*, 1911, vii, 1195.

¹⁶ Rolly, Fr., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 774; Kleinschmidt, *loc. cit.*

serum. This point, which is of high importance, is under examination at present so that a decision should soon be reached.¹⁷

Fortunately the number of strains or types of pneumococci appears not to be large. Neufeld and Händel¹⁸ have noted that most strains of pneumococcus are subject to the action of an immune serum prepared with a single culture of their type I pneumococcus, which is the culture employed for immunizing the goat and horse used in these experiments. Neufeld and Händel recognize four serum-specific types of pneumococcus, three of which are infrequent. It may happen that the specificity is not absolute with respect to the oleate serum mixture, and it is also, of course, possible that the obstacle to the usefulness of a single immune serum may be obviated by preparing a suitable polyvalent serum.

Other considerations which may affect the results of the employment of the oleate serum mixture are clinical in nature. From the experiments it appears that pneumococcic infections, started in the region of the brain, follow a severer course, and are less amenable to treatment than those started in the region of the spinal cord. In both, the experimental procedures produce traumatic injury and this injury gives to the infection a graver character when inflicted on the tissues of the brain than when inflicted on those of the cord. A class of pneumococcic inflammation of the meninges in man arises from infections of the membranes about the brain; namely, in middle ear disease, fracture of the base of the skull, infection of the accessory air sinuses, operations on the nasal tissues, etc. It remains to be ascertained to what extent this is a determining factor in the control of natural pneumococcus meningitis.

A great number of cases of pneumococcus meningitis in man follow blood infection with the pneumococci and are secondary to in-

¹⁷ Tentative experiments performed on rats and mice with two cultures of atypical strains of pneumococcus, obtained from the circulating blood of pneumonic patients, indicate that the atypical strains stand midway between the highly soluble typical pneumococcus and the insoluble *Streptococcus pyogenes* in respect to solubility in sodium oleate. The experiments indicate further that the atypical strains are resistant to the action of the mixture of typical antiserum, sodium oleate, and boric acid. Experiments are in progress to determine the effect of polyvalent immune sera which carry the specific antibodies for the atypical strains.

¹⁸ Neufeld, F., and Händel, *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 159; *Arb. a. d. k. Gsndhtsamte*, 1910, xxxiv, 293.

inflammations of the lung, pleura and heart, and arise generally throughout the meninges. In these cases the therapeutic control of the meningitis need not be equivalent to the suppression of the pathological conditions as a whole, to which the patient may still succumb. On the other hand, it is often just the concurrent meningitic infection that makes the general clinical conditions grave, so that with its abatement the outlook for the cessation of the other inflammation becomes much improved. Fortunately, the direct attack upon the meningitic infection is capable of affecting the blood infection since the eruption of pneumococci from the meninges into the blood is arrested while the ready escape of the therapeutic mixture from the meninges into the blood¹⁹ provides a favorable condition for the action of the immune serum upon the pneumococci already present in the blood, as well, possibly, as those situated in the interior of some organs.

The experiments show that the time interval between onset of infection and beginning of the specific treatment is a very important factor. The reasons for this are several: the degree of the infection and attending intoxication; the numbers of multiplying diplococci; the nature of the inflammatory exudate,—whether serous, purulent, or fibrinous,—that effects the penetration of the mixture to the diplococci and the remote parts of the cerebrospinal meninges. A purulent and fibrinous exudate is not only permeated less readily by the mixture, but its high protein content tends to reduce the action of the oleate upon the pneumococci upon which the efficiency of the mixture so much depends. Hence serous or seropurulent inflammations will probably be more subject to control than purulent and fibrinous ones. And yet in practice this distinction may make less difference than in the experiments in which the course of the infection is abnormally rapid. Should it be found that infection of the spinal meninges is sometimes controlled, while that of the cerebral meninges and ventricles is not, it will be well to consider the injection of the mixture directly into the cerebral ventricles, as has been successfully done in the serum treatment of epidemic meningitis.²⁰

¹⁹ Flexner, Simon, *loc. cit.*

²⁰ Cushing, H., and Sladen, F. J., *Jour. Exper. Med.*, 1908, x, 548; Fischer, L., *N. Y. Med. Jour.*, 1910, xci, 625.

Finally, the specific treatment described is not necessarily confined to meningitis but may be applicable to other local pneumococcal infections, such as those of the pleura, joints, and possibly the peritoneum. The determining conditions will be similar: the type of pneumococcus, the accessibility of the focus of infection (whether isolated by adhesions or communicating with the cavity into which the mixture is injected), the nature of the inflammatory exudate, and the coexistence of other slight or severe infections in inaccessible organs or parts. The oleate soap obviously possesses higher affinity or greater avidity for certain bacteria (e. g., pneumococcus) than for protein in general, since the boric acid suffices to hold it apart from serum protein under conditions in which it still attacks the bacteria.

CONCLUSIONS.

Virulent pneumococci injected into the cranial or spinal cavities of monkeys produce constantly a meningitis closely resembling pneumococcus meningitis in man, except that the experimental disease pursues a more rapid course to the invariable death of the untreated animal.

An homologous immune pneumococcus serum injected into the spinal canal exerts a restraining influence upon the disease to the extent that when employed early it prevented, exceptionally, the occurrence of infection and thus saved the life of the animal, and when given later it at first retarded the disease but subsequently exerted no beneficial action and was powerless to save life.

A mixture of sodium oleate, immune serum, and boric acid exerted regularly a more powerful action than immune serum alone, and not only prevented the occurrence of infection but also, when administered repeatedly, arrested the progress of an actually established infection and led, often, to the enduring and perfect recovery of the inoculated animal.

It is proposed to employ a similar mixture in the direct treatment of pneumococcal meningitis and possibly of still other accessible local pneumococcal infections in man.

CULTIVATION OF SPIROCHÆTA GALLINARUM.*

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATE 68.

Spirochæta gallinarum, discovered in 1903 by Marchoux and Salimbeni¹ in an epidemic in Brazil, is a septicemic organism responsible for a febrile disease of the chicken which often terminates fatally. The infection is transmitted by certain species of ticks (*Argas miniatus*, *Argas persicus*, *Argas reflexus*, *Ornithodoros moubata*), and the symptoms appear usually within three or four days after the tick's bite, and last for several days; there is no relapse. At the height of the fever the blood swarms with the spirochætæ, but in the event of recovery, they disappear within several days. *Spirochæta gallinarum* bears resemblance to *Spirochæta anserina*, discovered by Sakharoff² in the blood of geese and a spirochæta found by Balfour³ in a febrile chicken disease in Sudan, and Nuttall considers that the three forms are probably identical. According to Sakharoff, the anserina is incapable of infecting the chicken, while the gallinarum can infect not only the chicken, but sparrows, geese, and rabbits.

Spirochæta gallinarum bears a close morphological resemblance to spirochætæ causing relapsing fevers in man, but is somewhat more delicate than they. According to Novy and Knapp,⁴ the gallinarum measures about 0.25 of a micron in width and from 10 to 12 microns in length. In a fresh preparation of blood from infected birds it exhibits remarkable motility characterized by a swift forward movement and a lateral vibration of the entire body. It often forms a ring, turning first in one direction, then in the reverse direction.

* Received for publication, August 15, 1912.

¹ Marchoux, E., and Salimbeni, A., *Ann. de l'Inst. Pasteur*, 1903, xvii, 569.

² Sakharoff, M. N., *Ann. de l'Inst. Pasteur*, 1891, v, 564.

³ Balfour, *Reports of The Wellcome Research Laboratories*, 1908, iii, 38.

⁴ Novy, F. G., and Knapp, R. E., *Jour. Infect. Dis.*, 1906, iii, 291.

In some specimens when one end of the body is attached to the slide a swinging movement may be observed.

Spirochæta gallinarum has not previously been successfully cultivated outside the body. Borrel and Burnet⁵ observed an initial multiplication of the organism when a portion of the infected blood was mixed with chicken plasma, either pure or citrated, to which some bouillon was added afterwards; but no multiplication took place beyond the first tube. Levaditi⁶ observed multiplication for many generations by cultivating the spirochætæ in the peritoneal cavity of rabbits in collodion sacs containing infected blood.

In the course of my studies on the cultivation of spirochætæ of relapsing fevers,⁷ I undertook to cultivate *in vitro* *Spirochæta gallinarum*, and with success. I have now secured many generations by passing the culture from tube to tube at appropriate intervals.

Material.—Two different strains of *Spirochæta gallinarum* were employed. The first strain was kindly furnished by Professor Ehrlich in infected ticks from which the spirochæta was subsequently transferred to chickens. The infection of chickens by the ticks readily succeeded. The second strain of the gallinarum I owe to the courtesy of Dr. Levaditi of the Pasteur Institute of Paris. It was sent in infected blood hermetically sealed in glass tubes. Upon arrival, fifteen days later, a few motile spirochætæ were still seen under the dark-field microscope. The blood was first inoculated into canary birds and from the canary to the chicken.

For the purpose of cultivation the blood was aseptically taken under ether anesthesia from the heart of the infected birds and mixed immediately with a sufficient quantity of sodium citrate salt solution to prevent coagulation. The resulting emulsion of the blood which contained varying numbers of the spirochætæ was used for inoculating the culture media.

Culture Media.—The essential constituents of the culture media are: (1) fresh tissue, (2) ascitic fluid, and (3) paraffin oil. It is understood that the tissue, ascitic fluid, and paraffin oil must be absolutely sterile. The tissue must be perfectly fresh and of proper size.

⁵ Borrel, A., and Burnet, E., *Compt. rend. Soc. de biol.*, 1906, lx, 540.

⁶ Levaditi, C., *Compt. rend. Soc. de biol.*, 1906, lx, 688.

⁷ Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 176.

Kidneys of normal rabbits or pectoral muscles of chicken are best suited for this purpose. The ascitic fluid must be tested for its suitability, as many specimens are wholly unsuitable. The selection is to be made by actual cultivation experiment, which is done with several specimens of ascitic fluids simultaneously. This may seem very tedious, but when one finds good specimens, they can be kept on ice for future work and there will be no difficulty in continuing the cultivation for any number of transfers. The paraffin oil is best sterilized twice in an autoclave.

Technique of Cultivation.—Cultivation of this organism is simple and consists in placing a piece of fresh sterile tissue (about the size of a chestnut) and ascitic fluid in a sterile test-tube, followed by the introduction of several drops of the blood emulsion containing the spirochætæ. A layer of sterile paraffin oil is now poured in. The amount of ascitic fluid in the culture tube should be sufficient to reach a height of about ten centimeters. Incubation is at 37° C.

Mindful of the frequent occurrence of bacterial contamination, it is my custom to inoculate more than one culture tube on each occasion in order to obtain at least one pure culture. Bacterial contamination is a serious obstacle in the work, as when it occurs, it cannot be removed without passing the culture once more into chickens; sometimes the growth of the culture may be entirely suppressed according to the nature of the contaminating organisms.

Properties of Pure Culture.—*Spirochæta gallinarum* increases considerably in number within twenty-four hours of inoculation into the culture media. The rate of multiplication continues to be very rapid during the following three days, at which time several spirochætæ are found in each microscopic field. On the fifth day the growth comes to a standstill, and on or after the sixth day degenerative processes, indicated by reduction or loss of motility of many organisms and the irregularity of their curves, commence to prevail. While the majority of the spirochætal organisms undergo disintegration within ten days, a few motile ones are still present in the cultures kept at 37° C. on the fifteenth day, and very rarely is a living specimen present in cultures older than three weeks.⁸ The onset of

⁸ An exception to this rule has recently been met with while examining some culture tubes which had not been disturbed since inoculation thirty days previously and which had been continuously kept in the thermostat at 37° C. There were still quite a few motile spirochætæ.

the degenerative phase takes place gradually and is in striking contrast to the suddenness with which it occurs in the cultures of *Spirochæta duttoni*, *kochi*, *obermeieri*, and *novyi*.

The appearance of the culture media is but little modified by the growth of *Spirochæta gallinarum*, except that under certain conditions a very faint opalescence may appear. This happens especially when a piece of pectoral muscle of the chicken is used instead of rabbit kidney. No noticeable odor is produced in the culture.

No growth is obtained at room temperature or *in vacuo* at 37° C. Thus it is clear that the organism requires for its growth a certain amount of oxygen. In this respect the *gallinarum* resembles the *spirochætæ* of human relapsing fevers.

Subcultures.—Transplantation from culture to culture can be kept up for many generations and probably for indefinite periods, provided that a suitable specimen of ascitic fluid be employed. I have been able to continue cultivation of the two strains I worked with for fifteen successive transfers during the past two months. In continuous cultivation, the importance of suitable ascitic fluids cannot be too much emphasized.

Morphology.—(Figures 1 to 12.) In culture media containing a piece of fresh muscle of chicken and ascitic fluid, *Spirochæta gallinarum* presents all the morphological characteristics observed in the blood of an infected chicken. The organism measures about 0.3 of a micron in width and from 8 to 16 microns in length. Cultures three or four days old show numerous chains of two, three, or even four organisms. The organisms may be joined together by a short, thin filament that measures less than two microns in length, but sometimes the joint is merely suggested from the absence of curves, and there is no actual thinning. The number of curves varies according to the length of the specimen, but the distance between the curves is constant, measuring about 1.8 microns. Average curves have a depth of about one micron and usually a round apex. The position of the curves appears to change especially when the *spirochætæ* manifest violent movements. The regularity of the curves is most marked in the specimens which show moderate motility.* The

*The regularity of curves may be more or less lost during the process of drying on a slide, so that the stained preparation shows rather irregularly curved *spirochætæ* (figure 12).

movements are chiefly rotatory with more or less marked lateral vibration. The forward locomotion is less noticeable in the culture than in the blood. Agglomeration of spirochætæ is seldom observed even at the height of growth (usually on the fifth day) and in this respect the condition of culture differs from that of the blood of an infected bird.

Spirochæta gallinarum usually possesses a delicate, finely curved projection attached to one end of the body, measuring about three microns. It is rare to find a specimen with similar projections at both ends. In some specimens the projection is devoid of curves and appears to be more fragile than the curved ones. I am of the opinion that the straight projection is a prolongation of periblast and the curved one is continuous with the axial filament of the spirochæta. I arrived at this conclusion from my observations on cultured *Treponemata* (including the *pallidum*, *microdentium*, *macrodentium*, *mucosum*, *refringens*, and other relapsing fever spirochætæ).

Under somewhat less favorable cultural conditions certain atypical forms arise. These organisms may be short, having only two or three curves, or the curves may be irregular. This irregularity may also arise when a less suitable ascitic fluid is used and in a culture entering on the degenerative phase.

In all cultures one invariably finds varying numbers of round refractory bodies that are attached to the periblast of the spirochætæ. As a rule, one organism carries one such body which may be attached to a point anywhere along the side of the spirochæta. This spherical body measures about 0.75 of a micron. The number of the organisms with round bodies is inconstant in different cultures, but it seems to become greater where the growth of the culture approaches its maximum. Usually it is absent in cultures not older than two days. Apparently not every culture is capable of producing these bodies. Similar round bodies have been observed by previous investigators not only in the blood of the infected birds, but also in the fresh preparations of various other spirochætæ. I have described the presence of such round bodies in pure cultures of *Treponema pallidum*, *Treponema microdentium*, *Treponema macrodentium*, *Treponema mucosum*,¹⁰ *Spirochæta refringens*, *Spiro-*

¹⁰ Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 194.

chæta phagedenis,¹¹ *Spirochæta duttoni*, *Spirochæta kochi*, *Spirochæta obermeieri*, and *Spirochæta novyi*. The conditions under which the bodies are formed suggest that they represent one of the phases of the life cycle and should not be viewed as the result of plasmolysis, as some investigators have assumed.¹² At the same time they are certainly not the encystment forms described by Perrin,¹³ von Prowazek,¹⁴ and others in certain spirochætæ.

Another phenomenon observed in pure cultures of *Spirochæta gallinarum*, although common with all other spirochætæ studied by me in culture, is the formation of numerous granules in certain cultures, especially when their maximum growth has passed or the media have been somewhat defective. These granules are round or ovoid and are highly refractory. They do not exceed 0.3 of a micron and usually are about 0.2 of a micron in diameter. They show active molecular movements. They have doubtless been derived from the spirochætæ, since they are attached to numerous organisms along the entire body length. These granular spirochætæ may be quite actively motile or may show no sign of life. When the cultures are examined carefully, the granular spirochætæ are observed to be actually undergoing degeneration. On examinations day by day, these spirochætæ are seen gradually to give up the granules until they become completely or almost completely denuded, their immobile skeletal axial filaments alone remaining. In my opinion, the entire process is one of the degeneration, and the granules are nothing but the fragments of the periblast of the spirochætæ. Whether or not this phenomenon has any relation to the formation of coccoid bodies described by Balfour,¹⁵ Leishman,¹⁶ Bosanquet,¹⁷ Hindle,¹⁸ and others in blood specimens, I cannot say. At all events, the granules just described in cultures are incapable of

¹¹ Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 261.

¹² Hindle, E., *Parasitology*, 1912, iv, 463.

¹³ Perrin, W. S., *Arch. f. Protistenk.*, 1906, xii, 131.

¹⁴ von Prowazek, S., *Arb. a. d. k. Gsndhtsamte*, 1906, xxiii, 554; *Mem. do Inst. Oswaldo Cruz*, 1909, i, 79.

¹⁵ Balfour, A., *Jour. Trop. Med.*, 1911, xiv, 113.

¹⁶ Leishman, W. B., *Lancet*, 1910, i, 11.

¹⁷ Bosanquet, W. C., *Quart. Jour. Micr. Sc.*, 1911, lvi, 387.

¹⁸ Hindle, E., *loc. cit.*

causing the infection in chickens or of being cultivated in the media described.

While the morphological features of *Spirochæta gallinarum* in culture media containing chicken tissue remain typical for many generations, the organisms cultivated in media with rabbit tissue become somewhat thicker and the curves appear shallower. By transplanting these organisms back to the chicken media they once more become typical. On the other hand, the spirochæta grows just as abundantly in the presence of rabbit tissue as it does in the presence of chicken muscle.

In regard to the mode of multiplication I was able to observe unmistakable instances of transverse division by strangulation, but no longitudinal division could be detected. Whether or not the latter mode of division does not occur also under certain conditions must be determined later.

At no period of cultivation did bacillary forms appear, such as are described by Hindle¹⁹ as one of the phases of the life cycle of this organism in the tick.

Filterability.—In view of the findings of Novy and Knapp,²⁰ who caused an infection by injecting the filtrate of the blood derived from animals infected with *Spirochæta novyi*, I made several series of experiments in order to determine whether the cultures of *Spirochæta gallinarum* contained at any period a filterable form. For this purpose a mixture of cultures of varying ages (from two to thirty days) was filtered through several Berkefeld filters (V). The filtrate was tested in two ways. It was inoculated into chickens and cultured in suitable media. Controls for both series were provided with unfiltered cultures. Neither infection of chickens nor culture was obtained with the filtrates. In this connection I may mention that similar experiments performed with cultures of *Spirochæta duttoni*, *kochi*, *obermeieri*, and *novyi* gave negative results. These experiments do not affect the positive results obtained by Novy and Knapp, as they used filters which had been ground to lessen the thickness of the wall.

Pathogenicity.—*Spirochæta gallinarum* in culture does not lose

¹⁹ Hindle, E., *loc. cit.*

²⁰ Novy, F. G., and Knapp, R. E., *loc. cit.*

its virulence for the chicken to any noticeable degree when the transplantations are kept up at regular intervals in a suitable medium. Thus the cultures after thirteen transplantations caused fatal infection in chickens with characteristic symptoms of the disease. It remains to be determined by future experiments whether or not prolonged cultivation in artificial media will finally bring about an attenuation or abolition of virulence of this organism. I have found incidentally that when the transplantations into new media are neglected for about four weeks a culture which still shows numerous motile organisms no longer causes the infection in the chicken, even where a large quantity of the culture is inoculated.²¹ This seems to indicate that the *gallinarum* may become attenuated in virulence under certain cultural conditions; perhaps the attenuated cultures may confer immunity.

CONCLUSIONS.

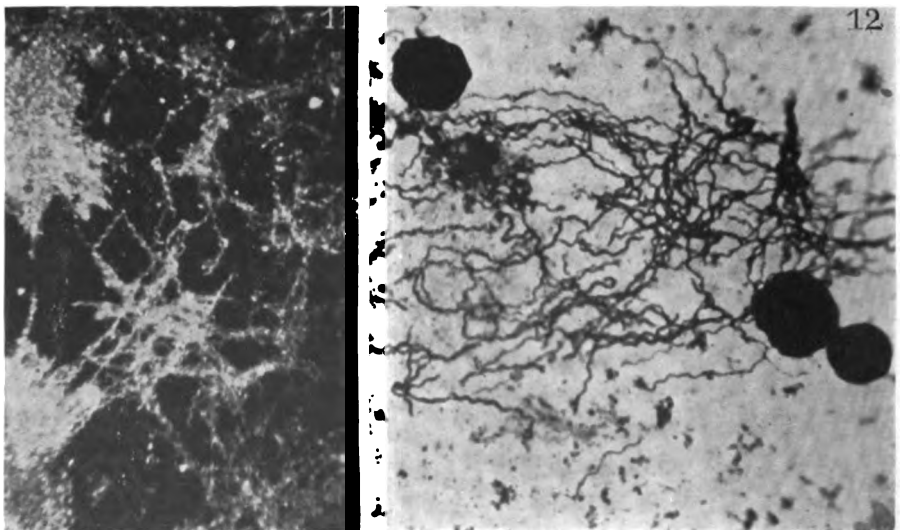
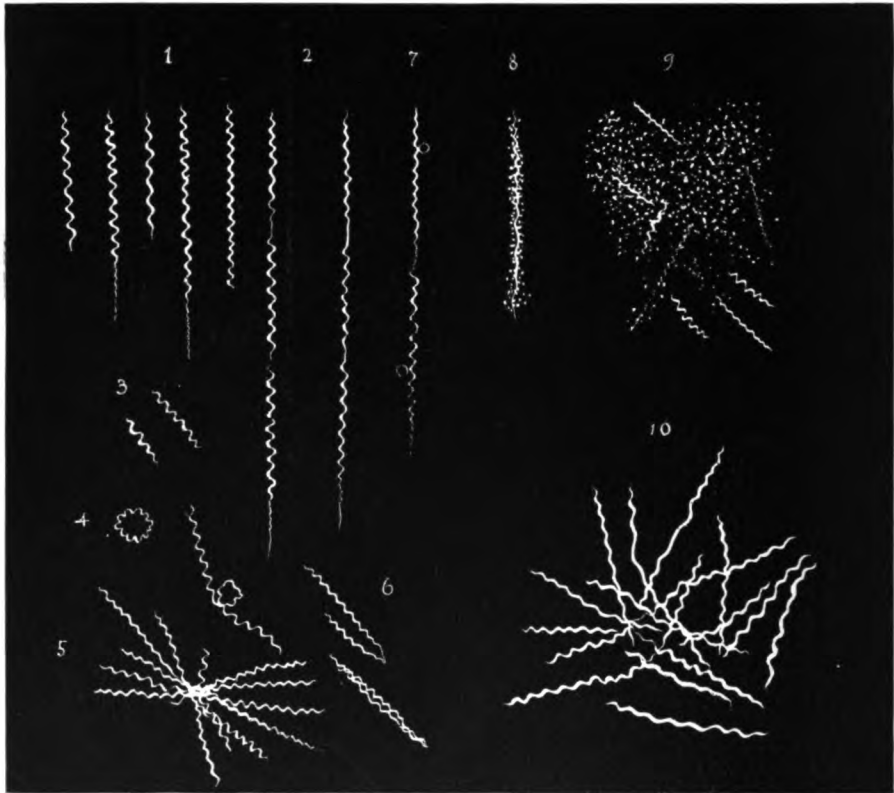
1. *Spirochæta gallinarum* can be cultivated in suitable artificial media for many successive generations and probably for indefinite periods. The presence of fresh tissue and a certain amount of oxygen seems to be essential for its growth. No perceptible odor is produced in the cultures.

2. The maximum growth of *Spirochæta gallinarum* is reached on about the fifth day, but the phase of degeneration commences slowly and gradually, so that in this respect the *gallinarum* differs from the *duttoni*, *kochi*, *obermeieri*, or *novyi*, whose cultures are characterized by sudden onset of degeneration soon after the maximum growth is attained.

3. No rod formation resembling bacilli arises in the course of multiplication of *Spirochæta gallinarum* in cultures. Many round or oval bodies appear in old cultures, but no infection of animals or formation of spiral forms from these granules has been produced. The granules are probably the degeneration products derived from the periblast of the spirochætæ.

4. Cultures of *Spirochæta gallinarum*, either old or young, do

²¹ The chickens which received the inoculation of the attenuated culture resisted the infection against a virulent strain directly from an infected bird when tested two weeks afterwards.



(Noguchi: Cultivation of *Spirochaeta gallinarum*.)

not contain a form which passes through a Berkefeld filter (V) that infects chickens or grows into spirochætæ.

5. *Spirochæta gallinarum* remains virulent for chickens after being in cultures for at least thirteen generations, but it may become avirulent under certain cultural conditions. The inoculation of chickens with the attenuated culture renders the birds refractory to the subsequent infection with a virulent strain.

6. When the spirochætæ are cultivated in the media containing rabbit kidney instead of chicken muscle, the individual specimens are somewhat thicker, but otherwise typical.

7. *Spirochæta gallinarum* multiplies in culture by transverse division. No positive evidence of a longitudinal division has been obtained.

EXPLANATION OF PLATE 68.

FIGS. 1 to 10. Schematic reproductions of *Spirochæta gallinarum* in pure culture, as observed under the dark-field microscope. Different stages of growth of the spirochætæ in a medium containing ascitic fluid and chicken muscle.

FIG. 1. Average forms; FIG. 2, long chains; FIG. 3, short forms; FIG. 4, ring formation; FIG. 5, agglomeration and knot formation; FIG. 6, doubled up forms; FIG. 7, spirochætæ with spore-like spherical bodies; FIG. 8, a spirochæta with granules; FIG. 9, degeneration phase with numerous granules, skeletal axial filaments, and several short motile spirochætæ.

FIG. 10. *Spirochæta gallinarum* in a culture medium containing ascitic fluid and rabbit kidney, showing somewhat thicker and less regularly curved forms.

FIG. 11. *Spirochæta gallinarum* from a pure culture (20th generation). $\times 1,100$. Dark-field view.

FIG. 12. *Spirochæta gallinarum* from a pure culture (20th generation) fixed in methyl alcohol and stained with the Giemsa solution for four hours. $\times 1,100$.

STUDIES IN INFARCTION.

II. EXPERIMENTAL BLAND INFARCTION OF THE LUNG.*

By HOWARD T. KARSNER, M.D.,
Assistant Professor of Pathology,

AND

J. EARLE ASH, M.D.,
Instructor in Pathology, Harvard Medical School, Boston, Mass.
(*From the Laboratory of Pathology (Phillips Fund), Harvard Medical School, Boston.*)

OUTLINE.

HISTORICAL NOTES.

SCOPE AND PURPOSE OF INVESTIGATION.

TECHNIC:

- Ligation of Bronchial Arteries.
- Ligation of Pulmonary Veins.
- Production of Artificial Pleural Effusion.

RESULTS:

- Simple Bland Embolism.
- Bland Embolism and Ligation of Bronchial Arteries.
- Bland Embolism and Ligation of Pulmonary Vein.
- Bland Embolism and Artificial Pleural Effusion.

DISCUSSION OF RESULTS:

- Congestion and Hemorrhage.
- Edema.
- Degeneration. Fibrin. Necrosis.
- Reaction in Tissues.
- Marginal Leucocytosis and Hyperemia.
- Decolorization.
- Regeneration.

CONCLUSIONS.

In a previous publication (1) a brief review of the literature of infarction in general was presented, little being said, however, about pulmonary infarction, a subject which in the present communication is discussed more fully because of the large number of clinical, pathological, and experimental investigations that have appeared.

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A most important topic, one that has received much attention (in spite of which there remains much to be learned), is that of the normal circulation of the lungs. One of the earliest careful investigations was that of Küttner (2), who showed that in the frog's lung the capillaries come off directly from relatively large vessels, that they are capable of taking over the function of anastomoses between two arteries or systems of arteries, that they can enlarge so as to resemble arterial anastomoses and further that veins with the aid of capillary streams can functionate as anastomoses between two arteries. These views were strongly supported by Stadelmann (3) from his pathological anatomical studies. Such free anastomosis had been denied by Rindfleisch (4), but the free anastomosis between bronchial and pulmonary circulation had been supported by Haller (5), Hyrtl (6), Henle (7), Luschka (8), and Virchow (9). Cohnheim and Litten (10) published their first study shortly after the appearance of Küttner's communication and claimed to have demonstrated by injection methods the interdependence of and lack of anastomosis between the bronchial and pulmonary systems. These findings, however, soon were contradicted by Küttner (11), who applied the methods of his earlier work to the study of mammalian lungs, reaching practically the same conclusions to which his work on the frog's lung had led. The validity of Küttner's work was acknowledged later by Litten (12), who stated that the earlier conclusions of Cohnheim and himself were faulty because of imperfect technic. On the publication of his "Vorlesungen" two years later, Cohnheim (13) gracefully accepted Küttner's conclusions. Küttner's work, however, is not without minor faults and the later work of Miller (14) would indicate that the intercommunication of the two systems is not so free as Küttner believed.

Nevertheless, there is sufficient reason adduced from these studies for the belief that simple occlusion of a pulmonary arterial trunk does not cause true infarction and many of the earlier workers accordingly declined to accept the view that pulmonary infarction was in any way related to embolism. It seems that Laennec failed to recognize the embolus as a cause of infarction, Rokitsansky (15) was not clear on the subject and Pannum (16) stated that the

pulmonary embolus produced no effect on the lung and was soon encapsulated. The experimental demonstration of embolism as the cause of pulmonary infarction came with the appearance of Cohnheim and Litten's (10) article and since then has been confirmed repeatedly, although not without some dissension, for as late as 1891 Grawitz (17) absolutely denied the embolic origin of lung infarcts and went so far as to state that if an embolus were present the infarct occurred in spite of it rather than because of it. This was in the face of the work of Klebs (18), who, by producing lateral conglutination thrombi in addition to the embolism, almost constantly obtained well marked infarcts. The later work of Fujinami (19), of Orth (20), and of Zahn (21) completely refuted Grawitz's conclusions and there could no longer be any doubt as to the causative relation of the embolus. Fujinami obtained results by injecting into the jugular vein paraffin of such consistence that the plugs would lodge not only in a large branch of the pulmonary artery, but also in several smaller branches, so as to shut off collateral circulation. Orth mixed various chemical irritants with the emboli and obtained fairly constant results. Although Küttner had noted that ligation of the pulmonary vein tended to make the infarcts more distinct, Zahn was the first to apply, what had been noted clinically, namely, that passive congestion increased the likelihood of pulmonary infarction and accordingly by tightly binding the rabbit's thorax, two days after the emboli had lodged, was able to produce typical infarcts in the lungs. If, however, he produced constriction earlier, the infarcts did not appear. It is generally known that the emboli in the vast majority of cases must lodge near the margin of the lungs in order to produce infarction. As far as can be learned the frequency with which the various lobes are affected has been reported chiefly by Tiedemann (22), who states that nearly fifty per cent of all infarcts of the lung occur on the right side at the base. In an analysis of thirty-three cases he found the right lower lobe involved in fifteen, the right upper in seven, the left lower in five, the left upper in four, and the right middle in two.

The circulatory disturbance produced by the embolus has been found to be variable with the size of the embolus or more literally

with the size of the vascular trunk occluded and also with the number of vessels occluded (work of both Fujinami (17) and of Klebs (18)).

Most workers have shown that the lodgment, if productive of any change, shows as a consequence the development of a generally conical area (affected in shape by the outer contour of the lung) of congestion, interstitial and alveolar edema and sometimes small foci of hemorrhage. Hemorrhage involving the entire area and subsequent tissue necrosis, however, is found but rarely unless some complicating condition is present.

The infarct of the lung is so typically hemorrhagic that the white or so-called anemic infarct is believed by many not to occur. In the experience of the writers, however, three such infarcts have been observed at the post-mortem table. There was marked pleural effusion and on histological examination the infarcts were found to be markedly necrosed and probably represented decolorized hemorrhagic infarcts. The possibility of such decolorization is discussed by Orth (23) and the occurrence of pallid infarcts has been noted by Welch (24), by Parson and O'Sullivan (25), and others.

The most noticeable feature of the infarct is the hemorrhage, which has received as much if not more attention than the infarct itself. Laennec, as is evidenced by the earlier name of the condition, apoplexia pulmonalis, considered it the most important feature and it is almost certain that Rokitansky (15) and others of even later times shared this view. That it is a secondary process, however, cannot be doubted, and the chief question is as to its origin. The general phases of the problem were discussed in an earlier paper (1), but certain special features in connection with the lung have received considerable attention. From the investigations of Virchow (9), Cohnheim (13), von Zielonko (26), and others, it is certain that lysis of the vessel walls in the area is associated with the hemorrhage, but as to its causative relation there is some question. There is unquestionably capillary dilatation and according to the views of von Zielonko (26), Brown-Sequard (27), Bier (28), and others, in general the hemorrhage is the result of increased vasomotor pressure. Much doubt, however, has existed as to the presence of vasomotor nerves in the pulmonary arteries, but

anatomically non-medullated nerves have been found in the dog's pulmonary artery (29), and the physiological work of Wood (30) and others can leave little doubt that vasomotor function exists. That increased capillary pressure plays an important part in the etiology of the hemorrhage might be inferred from the fact that it is almost impossible to produce hemorrhage in the lung infarcts unless passive congestion or some other circulatory interference is present, a fact well recognized both pathologically and clinically.

Necrosis is secondary to the marked circulatory disturbance produced by the embolism and hemorrhage, but is rarely seen at the autopsy table probably because of the fact that the patients usually are seriously ill at the time of the infarction and die before extensive necrosis occurs (see Welch (24)). This statement must hold true also in regard to decolorization of the infarct, because as Orth and Welch point out, and as is confirmed by our experience, although the condition is rare, it has been observed in human cases.

Conglutination of red corpuscles in the blood vessels and in the hemorrhage is an almost constant finding and was believed by Klebs to be important etiologically. Increased viscosity of the blood has been suggested by Funke (31) as being important from certain clinical studies, but no experimental work has been brought forward to confirm or contradict this view.

No studies on the healing of pulmonary infarcts have been made, but it is well known that completely organized conical areas, presumably healed infarcts, often have been found (Welch).

The purpose of the present work is to study the effects of experimental bland embolism and the consequent changes in the lungs, to investigate the effects of limiting the lung circulation, to devise a method for the constant production of infarction, and to study the changes found in the infarcts.

The alterations in general circulation were produced as follows: (1) For interruption of the bronchial artery supply a ligature was thrown around all the branches of the aorta below the left subclavian and including the second pair of intercostals. According to the general anatomists this includes all sources of bronchial arteries. Küttner claimed that branches might come from the internal mam-

maries and other arteries, but if such is the case the amount of blood so supplied would be inconsiderable because of the ramifications in the mediastinal ligaments. (2) For interruption of the pulmonary venous drainage, the branch coming from one lobe, in which a seed could be found by palpation, was tightly ligated. The venous drainage, in small degree, probably occurred through the bronchial veins. (3) Lesser degrees of congestion, as well as marked compression of the lungs, was produced by filling the thorax with sterile olive oil.

METHODS.

For the introduction of seeds into the pulmonary arteries, a piece of ordinary thin wall glass tubing was used and as an obturator a wooden applicator, such as is used by rhinologists, with a pledget of cotton on the end. The right jugular vein was opened aseptically under deep ether anesthesia, four turnip radish seeds placed in the end of the cannula, the cannula inserted from four to six inches toward the heart, and the seeds slowly discharged by means of the obturator. The vein was ligated and the skin wound closed with Michel clamps.

For ligation of the upper intercostal arteries, the anesthetic was administered by the Meltzer and Auer intratracheal insufflation method. The thorax was opened by resecting the left fifth and sixth ribs near the spinal column and the small branches of the aorta below the left subclavian (these frequently supplying bronchials), and including the first and second pair of intercostal were ligated en masse with a heavy silk suture thrown around by means of a large pedicle needle. The muscles were approximated with silk and the skin closed with clamps.

For the ligation of branches of the pulmonary veins draining lobes in which seeds were found, two methods were used. That of splitting the sternum under insufflation anesthesia was discarded because of the attendant shock. The second method was extremely satisfactory, the incision being placed in the seventh or eighth interspace and after cutting the intercostal muscles between the posterior axillary and parasternal lines on the left side a spreading retractor was inserted and the roof of the lung exposed. In all our cases so operated seeds were found in the left lung so that the operation did not need to be repeated on the opposite side. A silk ligature was thrown around the vein by means of a pedicle needle and tightly tied. In closing the wound one silk tension suture was thrown around the upper and lower ribs, by a full curved round pointed needle and the cut muscle brought to the lower rib by interrupted sutures carried through the muscle near the cut edge and around the lower rib. The external thoracic muscles were approximated with a single button-hole suture and the skin closed with skin clamps. The danger of pneumothorax was obviated by compressing the trachea about the insufflation tube and then compressing the chest before the last suture was tied. The method is that described by Quinby and Morse (32) and is much the same as that used by Carrel (33).

The olive oil was sterilized by heating twenty minutes in the autoclave at

twenty pounds pressure. The apparatus for injection consisted of a bottle with a two-hole rubber stopper into which were inserted two glass tubes, one of which was connected with a two-way trocar-cannula and the other connected with compressed air inlets supplying cotton filtered air under about ten pounds pressure. The oil was thus forced into the thorax at the rate of five hundred cubic centimeters in about twenty minutes. The amount of oil injected (under ether anesthesia) was determined by the appearance of gasping respirations and distinct smallness of femoral pulse.

All animals were killed with chloroform, the tissues fixed in Zenker's fluid, embedded in celloidin and stained with Delafield hematoxylin and eosin.

Bland Embolism and its Results.—Orientation experiments with various seeds showed that seeds smaller than the turnip radish seeds did not produce any grossly observable change in the lungs at the end of forty-eight hours. Therefore, not wishing to disturb pulmonary function, but to insure a sufficient number of emboli, four turnip radish seeds were injected into the superior vena cava and dogs killed one-half, three, six, twelve, twenty-four, and forty-eight hours, two, four, and seven days, three, four, and five weeks afterward.

At the end of one-half hour a sharply defined area of pallor was found, but no other gross changes. This area was generally triangular, the apex toward the point of lodgment of the seed, and was clearly visible in both the distended and collapsed lung. Such areas, as frequently was found in subsequent intrathoracic experiments, usually are about a third again as large as are the later areas of more marked vascular disturbance. Histologically, no changes were found other than very slight conglutination of the corpuscles in a few of the larger vessels.

At the end of three hours, however, changes very suggestive of infarction were visible. In the animal killed at this period two such areas were found, one in the sharp edge of the third right and lower left lobes. These areas extended two centimeters along the edge of the lung and the same distance into the lung, the apex of the doubly truncated cone appearing at the position of the lodged seed. On the outer surface they were well defined, slightly elevated, seemingly somewhat firmer than the normal lung and of dark red or crimson color with a very slight tinge of blue. On cut surface, the diamond-shaped area did not bulge but was sharply defined.

crimson in color, moist and bleeding. No areas of distinct consolidation were discerned. Histologically, a vaguely defined area was found in which there were well marked capillary dilatation and very distinct conglutination of erythrocytes in capillaries and larger vessels. The alveoli were of about normal size and almost universally filled with a clear, slightly acidophilic, homogeneous mass (edema), but there was no evidence of hemorrhage or desquamation. The interstitial tissue about the larger blood vessels was slightly edematous. The bronchi showed many goblet cells, but little other change. This area extended to within one or two millimeters of the pleura, the intervening lung tissue being normal, free from capillary distention, conglutination or any other changes described in the central area. The pleura was normal throughout.

At the end of six hours the same appearance was found grossly, but histologically there was beginning hemolysis, somewhat more noticeable interstitial edema and in the area immediately under the pleura, where no circulatory changes could be found, well marked distention of the alveoli, with apparent rupture of the alveolar walls but no hemorrhage.

At twelve hours the gross appearance was the same and histologically small areas of intra-alveolar hemorrhage were found, principally as a few corpuscles in the alveoli, but occasionally completely filling several alveoli.

At twenty-four hours the areas grossly showed a distinctly blue tinge in the red and histologically there were found in the larger vessels and in the edematous fluid of the alveoli numerous granules of golden yellow pigment resembling in every way hemosiderin granules.

At two days the same appearances were found grossly and histologically, and at four days no further changes were found.

At the end of one week the congestion was not so marked grossly and the area was correspondingly less well defined, but no notable changes were found histologically.

At the end of two weeks, instead of swelling, the area showed slight retraction on the inner surface, but not on the cut surface. Histologically, the air spaces had begun to open and there was considerable vesicular emphysema throughout the area. The peri-

bronchial and perivascular interstitial edema was by this time hardly discernible.

At three weeks no other notable changes were found grossly. Histologically, however, there was distinct thickening of the alveolar walls especially near the large connective tissue septa. No nuclear figures were seen, but the morphology of the cells in the latter position was that of fibroblasts.

At the end of five weeks the area was still somewhat, but very slightly, congested, swollen as if by emphysema, and poorly defined. On section, the outline was very sharp, the area somewhat more congested than on the outer surface and still moist. Histologically, there was little change except that the alveolar edema had almost disappeared and the thickening of the non-emphysematous alveolar walls was very well marked. Pigmentation had disappeared. A few capillaries showed conglutination, but in the vast majority the corpuscles were discrete. Most of the larger vessels were normal, but a few showed organization of thrombotic masses.

Throughout this series it was noted that the lodgment of seeds in those vessels whose terminals were directed toward a lung surface rather than an edge produced no noticeable change in the lung. The occlusion of vessels whose terminals were directed toward an edge or an angle of the lung almost invariably produced the changes indicated.

Conglutination was found histologically in most of the cases, but no subsequent changes were noted except slight hemolysis.

To summarize, it may be said that while an embolus of sufficient size produces definite and progressive circulatory and tissue changes in the lung, embolism is not in itself a cause of true infarction where one considers hemorrhage and necrosis as the criteria. Pallor of the embolic area was rapidly followed by congestion, swelling, and edema. These changes were followed histologically by granular pigmentation, emphysema, and fibrosis of alveolar walls.

Bland Embolism and Subsequent Ligation of Bronchial Arteries.

—In this series the dogs were killed one, five, seven, and fourteen days and four weeks after operation.

Grossly and histologically the changes were much the same as

those formed in Series I. except that congestion and edema were not so well marked either grossly or histologically. Emphysema was more prominent in the microscopic sections and the changes observed in the areas extended as far as the pleura instead of leaving a marginal area of normal lung tissue.

Bland Embolism and Subsequent Ligation of Pulmonary Vein.—

In this series the dogs were killed three, six, twelve, and twenty-four hours, two, four, and seven days, two, three, and four weeks after operation.

In the earlier cases, including the twenty-four-hour animal, the gross appearance of the area of embolic circulatory disturbance was the same as that seen in Series I. at the same periods except in size. As a result of the ligation of the vein, the congested lobe was considerably larger than normal and the embolic area measured three to four centimeters along the edge and extended approximately the same distance into the lung. Definition, swelling, color, and character of cut surface were the same as in the earlier series. By contrast with the deeply congested lobe the areas appeared lighter in color, but on comparison with areas in non-ligated lobes of the same lungs the color was found to be the same under both conditions.

At the end of two days, however, true infarction had developed. The area was sharply defined, elevated, solid, and of a very deep red, almost black color. On cut surface, it bulged slightly, was of the same color, sharply defined and of the texture and degree of dryness of a freshly cut relatively old blood clot. The non-infarcted portion of the lobe showed edema in addition to the congestion.

At the end of four days no gross change had occurred, but after one week the cut surface showed numerous dry, gray areas of necrosis, generally circular, situated near the middle and measuring one to two millimeters in diameter. At two weeks the necrosis was more extensive and still centrally distributed.

In three weeks the lobe had regained its normal size and grossly showed nothing but rich pigmentation. Correspondingly the infarct was smaller, measuring two centimeters along the edge of the lobe and extending fifteen millimeters into the lung substance. The infarct was firm, of the usual shape, distinctly and abruptly depressed, smooth and of a deep slate color. On cut section it was

well defined, slightly depressed, moist and of the same slate color, except that near the center was a small relatively dry, slightly protruding area, yellowish white in color and of pulpy consistence. The slate colored peripheral portion had a gelatinous sheen. At the end of four weeks no further gross changes had appeared.

Microscopically, the sections from animals killed before and including twenty-four hours showed almost the same appearance as in the non-ligated lobes and in Series I., the chief differences being the fact that the circulatory disturbance extended as far as the pleura, there was relatively little edema, but considerably more alveolar and interstitial hemorrhage when the venous outflow was obstructed. The non-infarcted lung showed much congestion and moderate edema. By the end of forty-eight hours, the hemorrhage had become very extensive, involving the entire lobe. In the infarcted area, however, it was extreme, the blood being packed tightly into the alveoli and markedly conglutinated, but this great degree of hemorrhage did not appear about the peripheral part immediately underlying the pleura, in which position, as with the earlier series, there was considerable emphysema. Desquamation of alveolar epithelium was moderate in the non-infarcted as well as the infarcted area, but in this period neither pigmentation nor phagocytosis was observed.

Numerous areas were found in the infarct where the alveolar walls and their nuclei had disappeared, but no such necrosis was observable grossly. The larger septa showed no degenerative changes and the large vessels although containing thrombi, both fibrinous and conglutinative, showed well preserved walls. The bronchi were filled with blood and fibrinous clot, but the epithelium remained normal. By the end of four days the densely packed hemorrhage had extended as far as the pleura, there were numerous fine golden yellow granules of pigment in the blood masses and edematous fluid and the large mononuclear phagocytes were filled with this pigment, but they appeared to show no migration to the lymph spaces. Conglutination was advanced and necrosis of the alveolar walls was emphasized by well marked karyorrhexis. Leucocytic infiltration was found in moderate degree about the margin between infarcted and non-infarcted lung, beneath the pleura and

about the large vessels within the infarcted area. Most noticeable at this stage was the active connective tissue proliferation in the same regions as indicated for leucocytosis. Nuclear figures were found in all three regions, but were most frequent beneath the pleura, in which position also endothelial proliferation and new capillary formation were most marked. The non-infarcted lung also showed connective tissue proliferation of less activity about the large vessels and under the pleura.

By the end of a week little change was to be seen except that leucocytes, fibrosis, and necrosis were more marked and decolorization of the blood masses appeared in circumscribed areas near the middle of the infarct.

At two weeks central decolorization was well marked, granular pigmentation was evident at the margin of the decolorized areas, and the fibrosis was considerably more marked than in the earlier infarcts. Nuclear figures in the connective tissue were less noticeable than in the four-day infarcts. Necrosis was complete in the central areas at this time. Edema had entirely disappeared.

At the end of three weeks the fibrosis had invaded the entire infarct except for a small area near the center, where open air spaces with heavy fibrous walls appeared. It evidently was this area which gave the gross appearance of necrosis in contrast to the more gelatinous connective tissue about it. Nuclear figures were still to be found, but were infrequent. A moderate amount of anthracotic pigment was present, but most striking was the rich hematogenous pigment present in macrophages scattered throughout the area but most marked near the periphery. The connective tissue of the pleura had become hyalin. The bronchi showed organization of the blood masses within them, numerous large macrophages containing blood pigment, but almost complete preservation of the epithelium.

At four weeks no other changes had appeared that were clearly distinguishable.

Bland Embolism and Artificial Pleural Effusion.—In this series the animals were killed at six, twelve, twenty-four hours, two and four days after operation. Other animals died eight, nine, ten, and eighteen days after operation. It seemed impossible to keep

the animals of this series alive a longer time because of the serious disturbances of nutrition following the injection of the oil.

Six hours after the operation the areas of embolic disturbance of circulation, in keeping with the reduced size of the compressed lung, were smaller than in Series I., usually extending about fifteen millimeters along the edge and the same depth into the lung tissue. The lungs were moderately congested and the embolic areas were seen as well defined pallid areas without elevation; the cut surface showed the same appearance and was moist. In the course of twelve hours, however, the areas were somewhat more congested than the surrounding lung, slightly swollen, and slightly bloody on cut section. No gross change was observed until the end of four days when the area was found to be of slaty blue color, sharply outlined, firm and depressed; on cut surface of same color, well defined, moist, slightly depressed. At the end of eight days the area was relatively smaller and on cut surface distinctly drier than the surrounding lung. In ten days, however, there was a splotchy central decolorized area which was drier than the surrounding infarct. At the end of eighteen days the central necrosis was more marked, but the infarct showed no other observable gross change.

Histologically, the production of infarction was not seen until eight days had elapsed. Before that the areas showed partial or complete collapse of alveoli, well marked capillary congestion, slight edema and moderate interstitial and alveolar hemorrhage. Conglutination was prominent and after twenty-four hours blood appeared in masses in the bronchi. At the end of eight days, however, the area was packed with conglutinated blood, the alveolar walls were well preserved, there was considerable pigment within the alveoli and a small amount within the alveolar walls, sometimes free, but usually within macrophages. The connective tissue of the pleura showed moderate proliferation as did also that around the large bronchi and blood vessels. No nuclear figures were found, probably because the animal had been dead for several hours before autopsy. After ten days the most notable change was the refilling with air of some of the centrally disposed air sacs, which showed markedly thickened walls. The blood near the center showed moderate decolorization. Most noticeable, however, was the

moderate leucocytic infiltration and extensive connective tissue proliferation, similar to that seen at the end of one week in Series III.

At the end of eighteen days no additional changes were found.

Discussion.—In reviewing the entire investigation, it can be seen clearly that simple embolism does not produce pulmonary infarction and that the added disturbance of bronchial circulation caused by ligating the upper branches of the thoracic aorta makes no change in the result. The pleural vessels are unaffected by the embolism and just as with the capsule of the spleen and kidney the vascular supply of the organ near the surface continues. It is only by slowing the circulation considerably as by the ligation of the pulmonary vein or by compressing the lungs that the infarction puts in its appearance. Granting that the artificial "effusion" produces less circulatory stasis of a given lobe than the complete ligation of its draining vein; and noting further that with the former procedure the infarct is not evident for from four to eight days, whereas with the latter it appears in forty-eight hours, it would seem assured that the degree of stasis is an important factor in the production of the lesion. Hence it may safely be said that not only is stasis a necessary corollary of the infarct, but also the greater the degree of stasis the sooner is the true infarction likely to appear. The length of time that elapses before hemorrhage becomes prominent would indicate that the pressure in the congested area is not such an important factor as the increased permeability of the vessel walls; which, since it appears at least several hours after the obstruction to venous drainage is produced, is most probably of degenerative origin. As in the kidney and spleen the hyperemia and hemorrhage appear in the order named. In the spleen hemorrhage appeared at four hours, in the kidney from twenty-four to forty-eight hours, and in the lungs at about forty-eight hours. The degree of capillary congestion in the embolic areas is about the same in both normal and congested lobes, but the capillary congestion is far more important in the lungs than in the kidney. Conglutination, prominent in the capillaries of the kidney and spleen, was by no means so prominent in the pulmonary capillaries.

Edema, both alveolar and interstitial, appeared in three hours and in the simple embolic process as well as in the lungs with addi-

tional ligation of the bronchial arteries, persisted to the end of the observation, becoming less marked as time passed. In the infarcts it disappeared in from one to two weeks, probably as a result of the extent and severity of the hemorrhage.

Simple embolic disturbance of circulation resulted in swelling of the alveolar epithelium at the end of three hours, which at six hours was followed by desquamation. In the cases where the pulmonary veins were ligated and in the cases where artificial effusion was produced, desquamation appeared at three hours. Fibrin formation was not evident before twelve hours, at which time it was found to be independent of the hemorrhage. Necrosis was not observed in the simple embolic process or with the added ligation of the bronchial arteries. In the true infarcts necrosis appeared in the alveolar walls at the end of forty-eight hours and became more extensive as the condition progressed, but did not invade the larger connective tissue septa carrying the bronchi or large vessels. Throughout all the series the bronchial epithelium remained well preserved. The gross evidence of necrosis appeared much later than the microscopic. Necrosis in the lung appears later and progresses less rapidly than in the spleen and kidney.

The tissue reactions in the simple embolic areas were not marked. At the end of three weeks there was a noticeable thickening of the alveolar walls near the larger connective tissue septa. Marginal hyperemia and leucocytosis were not noted. In the case of the true infarcts, however, there was seen at four days a well marked leucocytosis and fibroblastic reaction in the connective tissue of the pleura, in the line of demarkation of the infarct and in the larger septa within the infarct. Nuclear figures were clearly evident and the process continued most actively, so that at the end of four weeks organization was practically complete.

At two days, and more especially at four days, granular pigmentation appeared in all the series studied, much more markedly, however, in association with ligation of the pulmonary veins and with "effusion." Much of the pigment was in the bodies of mononuclear phagocytes apparently endothelial leucocytes. Pigmentation and phagocytosis of pigment are much more marked in the lungs than in

kidney and spleen, probably because of the greater hemorrhage in the lung.

Decolorization of the infarct began to appear both histologically and grossly at the end of one week as small irregular areas situated near the center of the infarct and later fusing so as to produce a large pallid central zone. The central zone of decolorization was not seen to reach the margin of the infarct at any time, almost certainly because the rapid ingrowth of connective tissue, with its richly pigmented macrophages, prevents marginal decolorization. The depraved condition of human patients with pulmonary infarction probably prevents such rapid organization, hence the complete pallor of old human pulmonary infarcts. The decolorization of the pulmonary infarcts appears later than that of the kidney and spleen infarcts, progresses more slowly and is never so complete. Essentially, however, it seems to be the same process in all three organs.

Although organization is rapid, there is no evidence of any attempt whatever at regeneration of either the alveoli or bronchi, the latter at no time showing any evidence of marked destruction. There was central emphysema in the infarcts of four and five weeks, apparently an opening up of old alveoli that had not completely undergone necrosis. This, however, cannot be regarded as a true regenerative process.

CONCLUSIONS.

1. Simple bland embolism of the pulmonary artery produces definite circulatory changes in the lung area supplied provided this area extends along the sharp edge of the lobe and the embolus is of sufficient size, but no evidence of true infarction is to be found.
2. So far as technical limitations permit, occlusion of the bronchial arteries makes no change in the circulatory alterations following simple embolism.
3. In the presence of embolism in a lobe, ligation of the pulmonary vein of that lobe leads to the formation of a true infarct, regardless of the position of the embolus. Artificial pleural effusion influences the embolic process in much the same way as ligation of the vein.
4. Infarcts so produced show the same early congestion, con-

glutination of corpuscles and edema as are produced by simple embolism, these changes being followed by cloudy swelling and desquamation of alveolar epithelium, hemorrhage and necrosis, decolorization and organization, the last proceeding from pleura, large connective tissue septa in the infarct and marginal non-infarcted lung. Marginal hyperemia, if present, is concealed by the general congestion of the lobe.

5. The pallor of the older infarcts is due to necrosis and decolorization of the contained blood; decolorization begins, in a general way, in the middle of the infarct and proceeds peripherally; decolorization is due in part to breaking up of the blood pigment, phagocytosis, and peripheral transportation, and probably in part to plasmatic diffusion.

6. Organization progresses rapidly, but no evidence of true regeneration is to be found.

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EXPERIMENTAL BRONCHOPNEUMONIA BY INTRA- BRONCHIAL INSUFFLATION.*

BY MARTHA WOLLSTEIN, M.D., AND S. J. MELTZER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATES 9 AND 10.

It is now generally assumed that the pathogenic organisms present in the lesions of the various forms of pneumonia form only one link in the chain of conditions which give rise to the development of these diseases. The natural or acquired specific resistance of the individual and of the species, the state of vitality of the individual and of the organ under consideration, are factors which, it is believed, determine the outcome of a bacterial invasion. This view was strengthened by the frequent failures in the attempts to produce pneumonias experimentally by using pure cultures of the corresponding microorganisms; and, in the case of bronchopneumonia, this view certainly seems quite plausible, since in most instances this form of pneumonia is associated with some other pathological condition. Recently, however, in forty-two out of forty-four dogs, Lamar and Meltzer¹ succeeded in producing lobar pneumonia with pure cultures of the pneumococcus. The injection of the cultures was made by a new method, that of intrabronchial insufflation. The two failures were due to some faulty step in carrying out the new technique. In the experiments of Lamar and Meltzer the animals were neither selected nor prepared in any manner, and the microorganism was, apparently, the essential factor in the causation of lobar pneumonia. The favorable outcome of these experiments suggested the advisability of applying the same method to the action of organisms usually found in association with bronchopneumonia.

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¹ Lamar, R. V., and Meltzer, S. J., *Jour. Exper. Med.*, 1912, xv, 133.

EXPERIMENTAL PART.

We have carried out a series of intrabronchial insufflations with the streptococcus and the influenza bacillus. In its essentials the method consists in introducing a tube through the mouth, larynx, and trachea, deeply into a bronchus, and then injecting the culture through the tube. The details of this method are recorded in the paper of Lamar and Meltzer.²

Experiments with the Streptococcus.—The virulence of several strains of streptococci isolated from cases of empyema and bronchopneumonia was tested in white mice, and the strain finally chosen was obtained at autopsy from the heart's blood and lungs of a fatal case of bronchopneumonia. When inoculated intraperitoneally in doses of 0.5 of a cubic centimeter of a twenty-four hour bouillon culture, this strain caused in twenty-four hours the death of a mouse weighing fifteen grams. Cultures of this organism were injected intrabronchially in twenty dogs in quantities varying from five to thirty cubic centimeters. In some experiments the cultures were enriched ("angereichert") by centrifugalizing them and resuspending the organisms. In this way the quantity of culture injected was made to contain two to six times the number of cocci present in the uncentrifugalized broth.

Of the twenty animals experimented with, one died within twenty-four hours. This was the only case in which the pneumonic lesion contained, besides the streptococcus, the bacillus of disemper. The other nineteen dogs were killed at intervals varying from twenty hours to six days. All had typical pneumonic lesions in various stages of progression and resolution depending upon the time elapsing between the injection and the killing of the animal. These lesions contained the streptococcus in pure culture.

The clinical course in these experiments was mild and of short duration and did not differ greatly from that described by Lamar and Meltzer for the non-fatal cases in the experimental infection with the pneumococcus. A few hours after the intrabronchial insufflation the temperature of the animal rose in some instances to above 40.4° C., but it came down, as a rule, on the following day and the animal soon appeared practically normal.

²Lamar, R. V., and Meltzer, S. J., *loc. cit.*

Lesions.—In the dogs killed from twenty to twenty-four hours after injection, the areas of consolidation were limited usually to the right posterior lobe, but in addition they were sometimes scattered in the subcardiac and upper lobes of the same lung, and were occasionally found in the other lung. The extent of the consolidation varied with the size of the dose injected; following a small dose the lesion involved sometimes only a small and superficial area, while after a large dose as much as half the lobe might be consolidated. The lungs were pink in color, contrasting with the dark red of the consolidated areas which were distinctly lobular in distribution, aerated lobules being discernible between the solid ones. The section was moist, smooth, and mottled, passing gradually by an irregularly ragged line into the aerated lung substance of the rest of the lobe. The pleura was not inflamed in any case, though its luster was impaired in several instances. In no case was there a central pneumonia. The solid areas and the lung adjacent to them were always deeply congested. Edema was slight. Smears from the solid areas showed many streptococci. Cultures from these areas gave a fairly profuse growth. From the uninvolved lung and from the heart's blood there was no growth.

After forty-eight hours the areas of consolidation were larger than after twenty-four hours, thus showing that the lesion was a progressive one. The dogs killed at the end of two days showed an extensive area of consolidation in the right posterior lobe, as much as two thirds or three fourths of it being solid, and usually there were consolidated areas in the right middle, left posterior, and left upper lobes also. The adjacent bronchial lymph nodes were but slightly swollen, were red in color, and were moist on section.

On the third day resolution was going on and the lungs showed consolidated areas of narrow and superficial extent only, the periphery shading gradually through a more or less congested area into the aerated lung substance.

On the fourth and fifth days resolution had progressed so that only a dark discoloration was left to outline the areas originally consolidated. In only one case was a narrow strip of quite solid pneumonia found on the sixth day, and to this animal the very large dose of thirty cubic centimeters had been given.

The right lung was involved in nineteen of the twenty cases. The left lung was also involved in eight instances, and in one case only was the pneumonia limited to the left posterior lobe. In twelve cases the areas of consolidation were scattered in two or more lobes of the lungs, and in a general way it may be said that the larger the quantity of culture fluid injected, the more scattered and the more extensive were the lesions; and the more numerous the organisms, the more severe the lesion. The enriched ("angereichert") cultures produced a more diffuse and intense inflammation, but did not delay resolution, for on the fifth day one case which had received a culture enriched six times was completely resolved. Streptococci were abundant in the smears from the consolidated areas on the first and second days and grew well in cultures. On the third day they were no longer profuse, and no growth was obtained on the fourth day. In no instance did the heart's blood give a growth of streptococci.

Microscopic Examination.—On microscopic examination within twenty-four hours the picture varied according to the size of the dose injected. In the least severe case the animal received only five cubic centimeters of culture. In this instance the exudate consisted chiefly of large mononuclear cells (the desquamated epithelial cells of the alveolar walls), with comparatively few polymorphonuclear leucocytes, and very little fibrin. The capillaries in the alveolar walls were intensely congested, but no red blood cells were present in the alveolar contents. The cells lining the bronchial walls were intact and no inflammatory infiltration was noted in these walls. The most densely packed solid alveoli were grouped around the smallest bronchioles, and in the areas which were the most solid, polymorphonuclear leucocytes were relatively more numerous than in the less solid areas, which, at the periphery of the lesion, shaded into lobules whose alveoli contained only granular coagulated serum and a few epithelial cells. Cocci were present in the alveoli in large numbers, and also in the bronchi,—many of which contained masses of exudate, evidently sputum.

A larger dose, fifteen cubic centimeters, had called forth a far more solid lesion in which the leucocytes were decidedly more numerous, and in which the walls of the smaller bronchi were distinctly

infiltrated. A still larger enriched dose, thirty cubic centimeters, had produced a very marked purulent infiltration, not only of the walls of the alveoli and bronchioles, but of the connective tissue septa, and even of the adventitia of the blood-vessels. There was very little fibrin present. The cocci were numerous.

On the *second* day the relative severity of the lesion had increased greatly, both in quantity and in quality. Thus the leucocytic infiltration had extended so that all the structures in a solid lobule were uniformly involved,—the alveoli, bronchi, blood-vessels, and septa—and the section gave a general impression of pus. This infiltration was most marked in the dog to which the largest dose had been administered. The desquamated epithelial cells were present in the alveolar exudate, but the leucocytes outnumbered them. Only a very small amount of fibrin could be detected. Beyond the solid areas some alveoli contained red blood cells, and all the capillaries were congested, as well as the smaller bronchial vessels. Cocci were still numerous, but phagocytosis was not marked.

On the *third* day resolution was progressing so rapidly that an area of bronchopneumonia caused by a small dose (five cubic centimeters) showed scarcely any packed lobules. There were very numerous examples of macrophage activity and a granular mass was present between the cellular remnants. In the more severe cases distinctly solid alveoli remained about a bronchiole, while the periphery of the lobules was becoming emptied of their exudate by phagocytosis and cell degeneration. Cocci were still present at this time.

On the *fourth* day only a few solid alveoli remained close around the bronchioles, and on the *fifth* day none were seen. Only a granular mass with a few leucocytes and epithelial cells was left in the alveoli, but the septa were still edematous and infiltrated with leucocytes, as were the alveolar walls. On the *sixth* day only one case was examined. This animal had received a large dose (thirty cubic centimeters) and in its lungs some well preserved exudate was seen in the alveoli around a bronchus, while almost all of the area that had previously been solid had undergone resolution.

There was no evidence of organization in any of these cases.

It is well to bring out here the difference between the pneu-

monia produced in the experiments of Lamar and Meltzer with the pneumococcus, and the pneumonia produced in the present series of experiments with the streptococcus. (1) In the pneumococcus infections there was a mortality of at least 16 per cent.; in our experiments there was practically no mortality. (2) In the pneumococcus infections there was a pneumococcus septicemia in all the fatal cases; no bacteriemia occurred in our streptococcus cases. (3) In the pneumonia lesions from the pneumococcus, the consolidated part of the lung was always air-free and lobar in character, no matter how small or large the affected area was; in the pneumonia lesions produced by the streptococcus infection, no matter how large the area, or how intense the inflammatory process was, aerated lobules were always discernible between the solid one; *i. e.*, the streptococcus pneumonia was "lobular." (4) In the pneumococcus pneumonia there was always a definite pleurisy present even in all the non-fatal cases, while it was practically completely absent in the twenty cases of the streptococcus infection. (5) In the pneumococcus infection the cut surface of the consolidated areas was rather dry and granular; in the streptococcus infection it was very moist and smooth. (6) Fibrin was an important element in the exudate of the lesions brought about by the pneumococcus infection, while it played practically no part in the pneumonia consolidation in our experiments. (7) In the pneumococcus pneumonia the walls of the bronchioles, although filled with exudate, were free from infiltration with leucocytes; while in the streptococcus pneumonia the bronchioles were intensely infiltrated by the leucocytes, especially in the more severe lesions. (8) Resolution progressed much more rapidly in the streptococcus pneumonia than in the pneumonic lesions produced by the pneumococcus.

The most essential points are the differences in the nature of the consolidation and in the fibrin formation.

ILLUSTRATIVE PROTOCOLS.

I. *Dog 2.*—Fox terrier, male; weight, 4,350 gm.

April 26, 1911, 12:30 P. M. Under ether anesthesia, a 5 c.c. dose of a twenty hour plain broth streptococcus culture was injected intrabronchially. 5 P. M. Temperature, 39.9° C.

April 27, 10 A. M. Temperature, 39.4° C.

April 28, 11:30 A. M. Temperature, 39.2° C. The animal at this time did not appear sick, but was killed with chloroform.

Autopsy.—The upper lobe of the left lung was normal. The posterior lobe was congested. The right posterior lobe was larger than any other, was swollen, and in its posterior third contained solid areas which involved the base also. There were a few very small and superficial solid areas in the right middle lobe. The pleura was quite normal. The bronchial lymph nodes on the left side were normal. On the right side they were a little enlarged, red in color, and very moist on section. On section the consolidated lung was smooth, patchy, red, and very moist. A thin red fluid exuded readily from the cut surface. The trachea was normal; the mucosa of the bronchi was reddened, and contained frothy, blood-stained fluid. Smears from the solid area showed numerous streptococci. In cultures from the fluid area a growth of streptococci developed, but there was no growth in the cultures from the heart's blood and from the left upper lobe.

Sections from the consolidated area showed that the alveolar exudate consisted of some large mononuclear (epithelial) cells and of a larger number of polymorphonuclear leucocytes, many nuclei being fragmented. The alveolar walls were infiltrated with these cells, and so were the walls of the small bronchi, the lumen being plugged with a mass of exudate. No fibrin was present.

II. *Dog 16.*—Yellow male; weight 4,800 gm.

June 6, 1911, 3 P. M. Under ether anesthesia, 30 c.c. of a twenty-two hour plain broth culture of streptococci, enriched to double strength, were injected into a bronchus. 6 P. M. Temperature, 40.4° C.

June 7, 9:30 A. M. Temperature, 39.7° C. 11:30 A. M. Animal killed with chloroform.

Autopsy.—The right posterior lobe was half solid and dark red, contrasting with the pink, aerated anterior portion of the lobe. The right apex and the subcardiac lobe also contained a solid area, and the upper half of the left posterior lobe was solid. The pleura was quite normal. On section the consolidated lung was mottled dark and light red, the light red areas being the smaller; they were aerated, while the dark ones were airless. Congestion and edema were very marked. Frothy fluid exuded from the bronchi. The bronchial nodes on both sides were congested and swollen.

Smears from the consolidated areas showed many streptococci. Cultures from these portions grew well; the heart's blood was sterile. Sections from the consolidated portion gave a general impression of pus; that is, the alveoli were filled with polymorphonuclear leucocytes and some epithelial cells. The leucocytes were numerous in the walls of the alveoli and in the bronchial walls; the smallest bronchioles showed degeneration of their lining cells, and the alveoli nearest to them were very densely packed with exudate in which there were a few short fibrin threads. All the vessels were distended with blood, but there were very few red blood cells in the alveoli.

EXPERIMENTS WITH THE INFLUENZA BACILLUS.

The relative frequency with which the influenza bacillus is found in cases of pneumonia led us to choose this organism for the production of experimental bronchopneumonia in dogs. The influenza bacillus may be present in pure culture in a human pneumonic lung, in which case the lesion is always of the lobular type. It may also be associated with the pneumococcus in lobar pneumonia, but it is found most frequently together with the streptococcus, the staphylococcus, or the pneumococcus in various combinations in cases of lobular pneumonia of the peribronchial type. It seemed a matter of some interest, therefore, to know how the intrabronchial injections of influenza bacilli would affect the lungs of the animal experimented upon.

A strain of the bacillus isolated from a case of leptomeningitis and virulent for rabbits and monkeys was used. It was grown on rabbit blood agar in Blake bottles and washed off in salt solution. The growth of one to three bottles, suspended in five to thirty cubic centimeters of salt solution, was injected. Eleven dogs were experimented upon and none died. It must be remembered that the dog has been found to be very resistant to influenza bacillus infection.

Within five or six hours after the injection of the culture, the dog's temperature rose to about 39.8° C.; in two cases it reached 40.2° and 40.7° C. On the following morning it had dropped to 39° or thereabouts, and then to 38.2° or 36.5° on the third day. In one case it remained about 39° C. for three days. Cough was not noticed and the animals were not apparently ill. They were killed at intervals that varied between twenty-one hours and six days after the injection.

The earliest lesion studied was twenty-one hours old. In this instance a small dose had caused scattered areas of consolidation in all the lobes of both lungs, but only one area was larger than 0.25 of a centimeter in diameter. The largest area was in the right middle lobe and was three centimeters in length. The consolidated part was not deep, but was very solid, though distinctly "lobular" in character. All the solid areas were deep red in color, and there were many hemorrhages in the aerated lung beyond. The pleura was normal.

A more concentrated dose produced in the same time a far more striking lesion. In this case the entire right posterior lobe was solid and small consolidated areas were scattered in the subcardiac and upper lobes as well as in all the lobes of the left lung. These areas were distinctly solid, were dark red in color, hemorrhagic, and moist, but small areas of aerated lobules were still visible between the solid areas. Smears showed many bacilli which grew in cultures. The heart's blood gave no growth.

Forty-eight hours after the injection of the organisms the lesion was of the same character, solid and dark red, not sharply demarcated from the aerated lung around it. Hemorrhagic areas were present in the lung.

In three days resolution was going on, the solid areas were resilient, were smaller in extent, and not so firm. On the fourth day the condition was about the same, while on the sixth day the pneumonia had almost entirely disappeared. At this time, after a concentrated dose, some resolved areas and a solid mass occupying half of the right posterior lobe were seen.

Microscopic Examination.—At the end of the first day the lesion showed that the exudate consisted of epithelial cells peeled off from the alveolar walls, some red blood cells, and many polymorphonuclear leucocytes. The alveolar walls were clearly outlined by the distended capillaries, and were not infiltrated. The bronchial walls were quite normal, and the lumen contained exudate composed of red and white blood cells, granular debris, and many bacilli. Only a very small amount of fibrin was present in the alveolar exudate. In the less solid portions of the lesion, the alveoli contained much granular serum, a few red cells, and some epithelial cells. In the same period of time (twenty-four hours) a more concentrated dose caused a more marked leucocytic exudation. Many of the leucocytes with fragmented nuclei infiltrated the bronchial and alveolar walls. There was very little fibrin; many bacilli were present, and hemorrhages into the alveoli near the solid areas were very numerous.

At the end of the second day the exudate was even more markedly leucocytic in character, and a purulent infiltration was present in all the bronchial walls, interlobular septa, and the alveolar walls. All

the bronchi contained plugs of exudate and around the bronchi were densely solid alveoli. On the third day resolution had begun. The congestion had subsided, the phagocytic cells were numerous in the exudate, and all the leucocytes were undergoing fragmentation. Bacilli were still very numerous.

On the sixth day the lesion produced by a small dose was almost entirely resolved, some granular serum and cell debris remaining in some of the alveoli, while the purulent infiltration had subsided. A more concentrated dose had produced a lesion which, on the sixth day, showed resolving pneumonia, as well as large peribronchial areas of consolidation in which resolution had not even begun.

There was no pleural exudate in any one of these eleven cases. Cultures of influenza bacilli were obtained from the pneumonic areas on the first, second, and third days, but from the heart's blood no growth was obtained at any time.

In the influenzal bronchopneumonia, as in the streptococcal variety, the size of the dose of bacteria injected had a distinct influence on the pneumonic areas that developed. The most extensive and most severe lesions were produced by a larger and enriched dose, and in such a case resolution was also delayed as compared with that of a lesion caused by an injection containing fewer organisms. Hemorrhage and purulent infiltration are characteristic features of this variety of lobular pneumonia.

ILLUSTRATIVE PROTOCOLS.

I. *Dog 27*.—Black and white female; weight 3,700 gm.

June 6, 1911, 3 P. M. Under ether anesthesia, 10 c.c. of a suspension of influenza bacilli which had been grown in two bottles for twenty-four hours were injected intrabronchially. 6 P. M. Temperature, 40.2° C.

June 7, 9:30 A. M. Temperature, 39.6° C. 11:30 A. M. The dog was killed with chloroform.

Autopsy.—The posterior lobe of the right lung was swollen, large, solid, firm, and dark red. The entire lobe was involved. On section, this lobe was hemorrhagic, solid to the touch and moist, and revealed very few aerated lobules among the solid areas. The greater part of the lesion, however, was hemorrhagic rather than inflammatory. The pleura was normal. On the right upper lobe were scattered areas of consolidation. These were also present in the subcardiac and middle lobes. In the left lung there were solid areas in all the lobes. These solid areas were larger near the upper angle of the posterior lobe, and all of them were peribronchial and interspersed among the aerated lobules. The bronchial nodes were deeply congested and very moist.

Smears from the right posterior lobe showed many leucocytes and many Gram negative bacilli. The influenza bacillus grew in pure culture.

Microscopical Examination.—The alveoli were filled with peeled off epithelial cells, polymorphonuclear leucocytes, and some red blood cells; there was some granular fibrin in the exudate, but it was nowhere excessive. The smallest bronchi had lost their epithelial lining and there was leucocytic infiltration of their walls. The larger tubes, however, had normal walls, but contained aspirated masses of leucocytes and red blood cells. Empty alveoli were present next to solid lobules. Bacilli were very numerous in the alveoli and bronchi. Many alveoli at the periphery of the solid areas were filled with red blood cells, and many other alveoli contained serum only.

II. *Dog 29.*—Black male; weight 7,400 gm.

June 13, 1911, 11 A. M. Under ether anesthesia a 10 c.c. suspension of the twenty-four hour growth from three bottles of influenza bacilli was injected intrabronchially. 5 P. M. Temperature, 40.2° C.

June 14, 9:30 A. M. Temperature, 39.6° C.

June 15, 9:30 A. M. Temperature, 38.8° C.

June 16, 9:30 A. M. Temperature, 38.7° C.

June 17, 9:30 A. M. Temperature, 38.5° C.

June 19, 9:30 A. M. Temperature, 38.9° C. The animal was killed with chloroform.

Autopsy.—The only consolidation found was in the right lower lobe. This was about half solid, and beyond was a dark area which was evidently almost resolved. The consolidated portion was firm and smooth, and on section was very moist and of a greyish red color. The dark area was aerated and more congested than the rest of the lobe. All the remainder of both lungs was quite normal. The lymph nodes on the left side were normal. On the right side they were slightly swollen.

Smears and cultures were negative for influenza bacilli. Sections from the consolidated area showed that the alveoli were filled with epithelial cells and polymorphonuclear leucocytes. No fibrin was present. The capillaries in the walls were not distended, but leucocytes were present between the meshwork. All the bronchi, large and small, showed extensive degeneration of their lining cells and a leucocytic infiltration of their walls, this infiltration being continuous in places with the exudate in the surrounding alveoli. At the periphery of the consolidated area there was marked phagocytosis of the cell fragments by large mononuclear cells. In some cases whole red blood cells or leucocytes were contained in these macrophages. As the cells diminished in number a granular material occupied the alveoli, and there was a gradual transition from these areas to those of normal empty air cells. No bacilli were found in these sections.

The differences between the lesions produced by the injection of the pneumococcus into the bronchi and those caused by the influenza bacilli were as marked as were the differences between the lesions produced by the pneumococcus and those caused by the streptococcus. The fibrin was slightly larger in amount in the influenzal

pneumonia than in the streptococcal variety, but was by no means as marked a feature of the exudate as in the pneumococcus pneumonia. Hemorrhage into the alveoli was more marked in the influenza cases than in either the streptococcus or the pneumococcus lesions, and leucocytic infiltration was as severe. In gross appearance influenzal bronchopneumonia was distinctly patchy and lobular, not lobar in type, although the tendency was for a large portion of a lobe to be involved.

SUMMARY.

When intrabronchial insufflation of pure cultures of the streptococcus or of the influenza bacillus is properly carried out, it produces without fail a pneumonic lesion. This lesion is similar in its nature to the one known in human pathology as bronchopneumonia, and differs materially from the pneumonic lesion produced experimentally by the intrabronchial insufflation of pure cultures of the pneumococcus.

Considering the fact that none of the dogs used in the experiments with the pneumococcus and none of those used in the present investigation were selected or prepared in any way, the conclusion seems to be unavoidable that the proper invasion of the microorganism is the determining factor in the development of pneumonia, the condition of the animal being only a minor element in this regard.

Furthermore, since different organisms introduced in the same way and under conditions which are apparently the same produced distinctly different pneumonic lesions in animals of the same species, the further conclusion presents itself that the different types of pneumonia are produced by specifically different bacteria.

However, further investigation may show that the differences in the nature of the lesion are due rather to the degree of virulence of the causative microorganism than to differences in the species; that is, that different lesions may possibly be produced by organisms of the same species, provided they possess different degrees of virulence. Further experimentation may also show that the condition of the animal and of the affected organ which, in the onset and development of the pneumonic disease, is, perhaps, unimportant, may be the leading factor in determining the course and outcome of the disease.

EXPLANATION OF PLATES.

PLATE 9.

FIG. 1. Streptococcal bronchopneumonia of seventy-four hours' duration; dose 10 c.c.; twenty-four hour bouillon culture.

FIG. 2. Streptococcal bronchopneumonia of forty-eight hours' duration; dose 10 c.c.; enriched three times.

PLATE 10.

FIG. 3. Influenzal bronchopneumonia of twenty-four hours' duration; dose 10 c.c.; enriched twice.

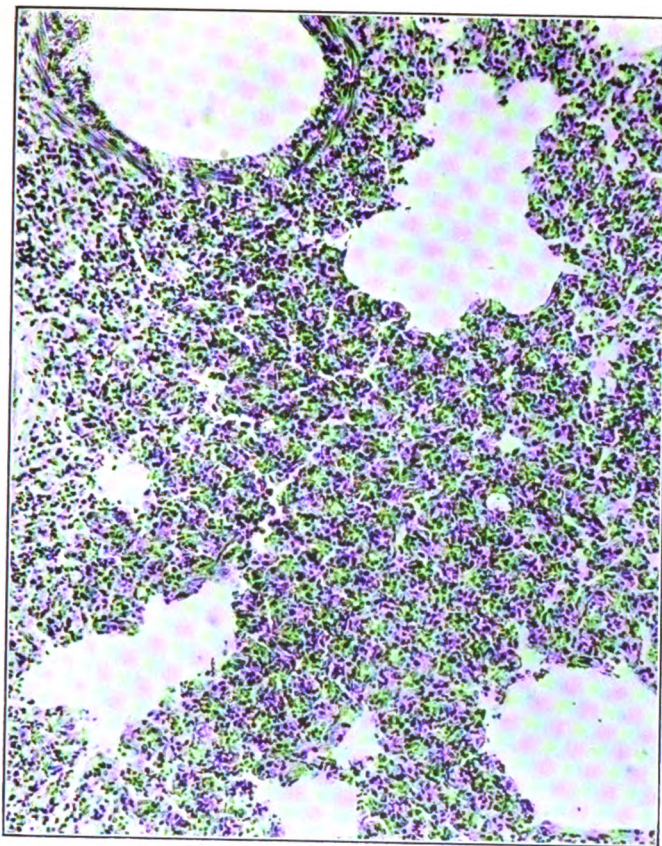


FIG. 1.

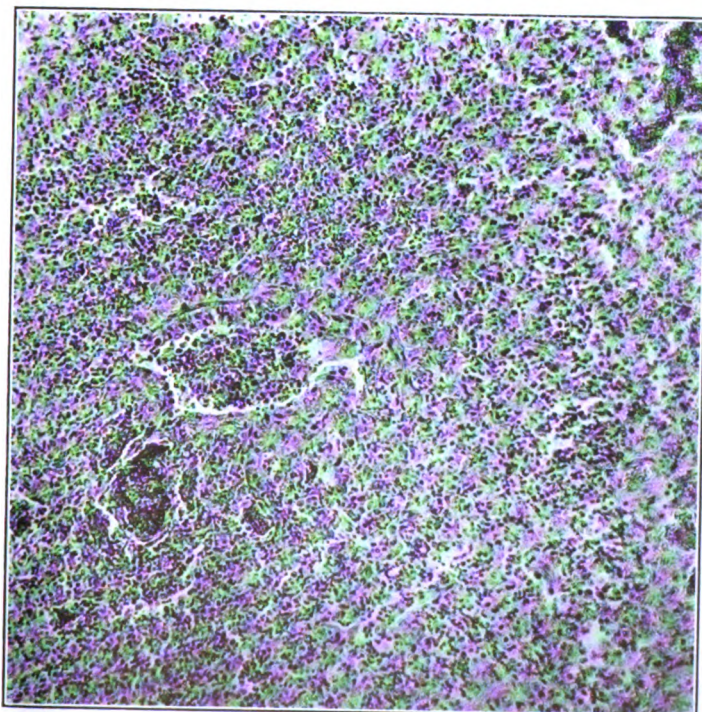


FIG. 2.

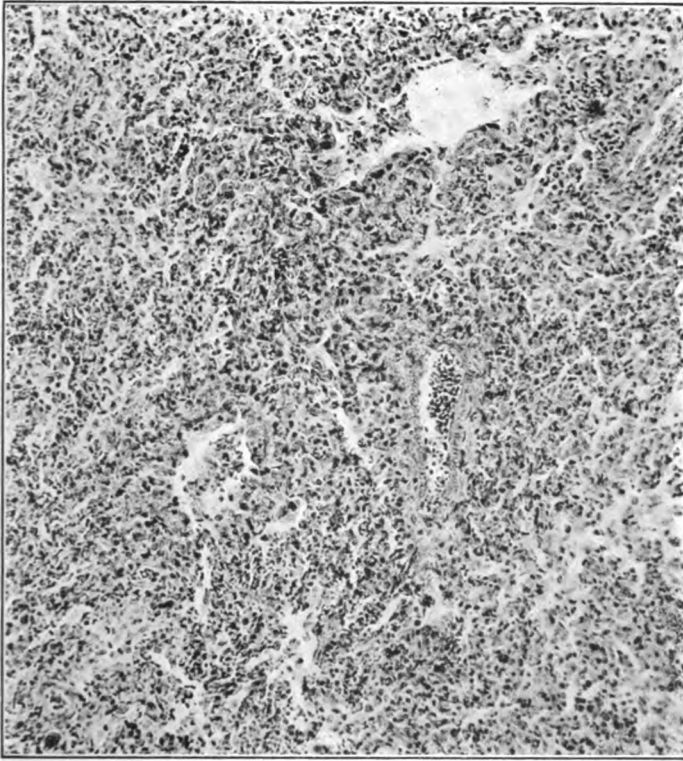


FIG. 3.

ON THE ACTION OF LEUCOCYTES ON GLUCOSE.

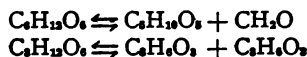
SECOND COMMUNICATION.*

By P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

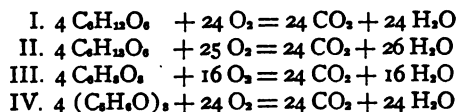
The results reported in a previous communication¹ were convincing of the fact that lactic acid is formed through the action of leucocytes on glucose. However, the quantity of lactic acid isolated in form of its zinc salt was lower than that of the disappeared sugar. Hence, to complete the knowledge of the action of leucocytes on glucose it was necessary to ascertain the fate of the missing sugar that had not been identified as lactic acid. The theory admits of a great variety of possibilities and an attempt was made to test by experiment some of them.

The disappearance of sugar may be caused by either one of the three reactions, oxidation, reduction or dissociation, such as



It was therefore concluded to inquire into the type of reaction which caused the disappearance of the missing sugar under the influence of leucocytes, without attempting to isolate the products of the reaction. A means for distinguishing the three types of reactions is found in the fact that the products formed in each one of them require a different quantity of oxygen for transformation into carbon dioxide.

Thus:



* Received for publication, July 2, 1912.

¹ This *Journal*, xi, p. 361, 1912.

It is seen from this that taking glucose for the unit value of oxygen requirement for oxidation into carbon dioxide, a higher value is needed for oxidation of an equal number grams molecule of the reduction products, a lower value when oxidation was responsible for the disappearance of sugar and an equal value when the breaking down of sugar was caused by a reaction of dissociation.

Recently Greifenhagen, König and Scholl² have determined the conditions for oxidation of several carbohydrates, of methyl alcohol, and of glycerin into carbon dioxide by means of a potassium permanganate solution. The authors recommend the method for quantitative estimation of sugar.

It was concluded to resort to this method for estimation of the oxygen requirement for transformation into carbon dioxide of the products formed by the action of leucocytes on glucose. However, before applying it, the method was tested in regard to the following requirements:

1. Whether it permits of detecting accurately such small differences in sugar concentration as occur in course of our leucocyte experiments.
2. Whether it permits of detecting saccharic acid in such concentrations as may occur in the leucocyte experiments.

The method was found to satisfy these two requirements and it was therefore applied to the analysis of the products of reaction of leucocytes on glucose.

It was found that while the reducing power of the sugar solution suffered the usual loss of about 10 per cent., the oxygen consumption of the original sugar remained unaltered at the end of the experiment. A control of the leucocyte extract treated in the same manner as in the principal experiment did not demonstrate any estimable reducing power on permanganate solution. On the basis of these experiments one was forced to the conclusion that the products of the action of leucocytes on glucose are neither oxidation nor reduction products.

It also was known from the results of previous experiments that the substances formed in the reaction were not volatile and did not possess reducing power for Fehling's solution.

² *Biochem. Zeitschr.*, xxxv, p. 169, 1911.

The possibilities that suggested themselves on the basis of these data were, either that all the missing glucose was transformed into lactic acid, or that part of it was transformed into glyceric aldehyde which in its turn suffered Cannizzaro's transformation into glyceric acid and glycerol. The attempts to detect glycerol in our reaction products were not successful.

These considerations seemed to point to the conclusion that all the missing sugar was transformed into lactic acid and yet only about 40 per cent. of the missing sugar was extracted in form of the acid. Thus we were led once more to test the efficiency of our method of extracting lactic acid. In the previous communication mention was made of the fact that about 90 per cent. of lactic acid was recovered when the acid was added to a suspension of leucocytes and the mixture was treated in the same manner as the reaction mixture of the principal experiments. In the course of the present work an experiment was made with a view of establishing the action of leucocytes on lactic acid. In that experiment the lactic acid was allowed to remain in contact with the leucocytes for thirty-six hours at 37° C., and in that instance nearly all the added lactic acid was recovered by extraction. This experiment may also serve as a test of the efficiency of estimating lactic acid by the extraction method. However, there were lacking experiments testing the efficiency of the extraction of lactic acid from mixtures containing only about 10 per cent. of the acid and 90 per cent. of glucose. Buchner and Meisenheimer³ have made such experiments with mixtures of lactic acid and saccharose and have regained by extraction about 90 per cent. of the acid present in the mixture. We have repeated their experiments and have obtained as good results as they. However, when such experiments were performed with mixtures containing glucose instead of saccharose, only about 40 to 50 per cent. of the lactic acid present in it were extracted—approximately the same values as those obtained in our principal experiments. This is due perhaps to the fact that the physical nature of the glucose mixture does not permit of a very intimate mixing with the sodium sulphate powder and that, in its turn, makes the extraction of lactic acid imperfect. Thus the extraction method

³ *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 1784, 1910.

can serve only to identify the lactic acid formed in course of the experiment and cannot be relied upon for its quantitative determination. For this, use has to be made of the oxidation method by means of permanganate solution. A test experiment showed that the values obtained by the oxidation method are identical with those obtained by titration. On the basis of these considerations we feel justified in the conclusion that lactic acid is the only product of the action of leucocytes on glucose.

CONCLUSIONS.

1. Under the influence of leucocytes glucose undergoes dissociation into lactic acid only.
2. The lactic acid formed under these conditions remains intact.
3. There is no evidence of the formation of any oxidation products of glucose in the same experiments.
4. It is remarkable that under conditions approaching those existing in the animal body the transformation of glucose is so simple and uniform while the formation of lactic acid from glucose by purely chemical methods is always accompanied by numerous side reactions.

EXPERIMENTAL PART.

The technique of the experiments was the same as in the preceding communication. The details of permanganate titration method are as follows:

The protein-free sugar solution was made up to a definite volume and a quantity containing approximately 30 mgm. of sugar used for each oxidation. This was accomplished by boiling with 40 cc. of $N/5$ permanganate solution and 60 cc. of 10 per cent. potassium hydrate in a round bottom, long necked, 500 cc. Jena flask, fitted with a long wide tube to prevent loss of liquid by possible bumping. Bumping was kept at a minimum by adding a sufficient quantity of glass pearls and boiling vigorously over a wire gauze. Boiling was continued for ten minutes.

To the hot solution were then added 50 cc. of 25 per cent. sulphuric acid and sufficient $N/5$ oxalic acid solution to completely decolorize the solution which was titrated back to pink with $N/10$ permanganate solution. The amount of permanganate used for the oxidation of the sugar is the difference between the total $N/5$ permanganate solution (cc. $N/10$ permanganate reduced to cc. $N/5$) less the number cc. $N/5$ oxalic acid added. The determination takes about twenty minutes.

Total Oxidation of Sugar by Method of Greifenhagen, Showing Differences of 1 mgm. Sugar.

Different volumes of a 2 per cent. solution of glucose were oxidized by the process described earlier in this paper. The results are presented in the following table. All reported values represent averages of several determinations.

GLUCOSE SOLUTION	GLUCOSE	$\frac{N}{5}$ KMnO_4	$\frac{N}{5}$ OXALIC ACID	DIFFERENCES	DIFFERENCES FOR 1 MGM.
cc.	mgm.	cc.	cc.	cc.	cc.
15.0	30	40.99	21.03	19.96	
15.5	31	42.48	22.10	20.38	0.42
16.0	32	43.82	22.75	21.07	0.68
16.5	33	43.88	22.10	21.78	0.71
17.0	34	44.18	21.70	22.48	0.70
					Average: 0.6275

Calculated difference for 1 mgm. glucose = 0.666 cc.

Oxidation of Saccharic Acid.

Acid potassium saccharate was prepared in the laboratory by the usual process. The crude salt was recrystallized several times before it was used for the experiment. It was dried to constant weight in a vacuum desiccator over phosphorus pentoxide. 0.496 gram of the salt was dissolved in 100 cc. of water. Of this solution 10 cc. were employed for oxidation with 40 cc. of $N/5$ permanganate solution and 60 cc. of a 10 per cent. solution of potassium hydroxide. For decoloration it was necessary to add 23.5 cc. of $N/5$ oxalic acid. The colorless solution was titrated to permanent pink color by 2.0 cc. of $N/5$ permanganate solution; it required 18.5 cc. of $N/10$ permanganate solution to oxidize 0.0496 gram of acid potassium saccharate.

The equivalent amount of glucose requires for oxidation 24.9 cc. of permanganate.

Oxidation of Mixtures of Saccharic Acid and Glucose.

For this series of experiments two solutions were prepared: (A) 30 mgm. glucose dissolved in 250 cc. of water and (B) 41 mgm. acid potassium saccharate dissolved in 250 cc. of water. Twenty-five cubic centimeters of each of these solutions were oxidized sep-

arately according to the method previously described. A mixture of these solutions consisting of 23.30 cc. of the glucose solution and 1.64 cc. of the acid potassium saccharate solution was also subjected to this process. The fractional parts of a cubic centimeter were measured off on a specially graduated pipette.

The following table summarizes the results.

SOLUTION	GLUCOSE (A) OR SACCHAR- ATE (B)	N $\frac{1}{5}$ KMnO ₄	10 PER CENT KOH	ADDI- TIONAL N $\frac{1}{5}$ KMnO ₄	N $\frac{1}{5}$ OXALIC ACID	DIFFER- ENCE N $\frac{1}{5}$ KMnO ₄
cc.	mgm.	cc.	cc.	cc.	cc.	cc.
25.00 (A)	30.0	40	60	2.9	22.5	20.6
25.00 (B)	41.0	40	60	0.7	25.5	15.2
23.30 (A)	28.0	40	60	3.8	24.1	19.7
1.64 (B)	2.2					

Oxidation of Leucocytes and Phosphate Solution Alone.

For this series of experiments there was prepared a mixture consisting of a leucocyte suspension and of a Henderson phosphate solution combined in the same proportions as employed in the glycolysis experiments. The mixture was allowed to stand at 37° C. for thirty-six hours.

The proteins were then removed by coagulation and subsequent filtration. Twenty-five cubic centimeters of the filtrate were boiled with 40 cc. N/5 permanganate solution and 60 cc. of a 10 per cent. solution of potassium hydroxide. For decoloration were employed 45.0 cc. of N/5 oxalic acid. The excess of oxalic acid was oxidized by 6 cc. of N/5 permanganate solution. Hence 1 cc. of N/10 permanganate solution was required to oxidize 25 cc. of the tested solution. Considering that the 25 cc. of the filtrate used is five times the quantity used in the sugar oxidations, the possible error in those determinations due to organic matter in the leucocytes would be equal to 0.2 cc. N/5 permanganate solution, which amount is insignificant.

Action of Leucocytes on Glucose.

In this series of experiments the reduction values for Fehling's solution were compared with those for a permanganate solution. The comparative estimation was made at the beginning and at the end of the experiments. A suspension of sterile leucocytes in

a Henderson phosphate solution containing sugar was allowed to stand thirty-six hours at 37° C. The reduction of the Fehling's solution was measured by Volhard's method and of the permanganate solution by the process described earlier in the paper.

The reducing power of the solution was also tested after the glycolized solution had been boiled for two hours with a 2 per cent hydrochloric acid solution. This experiment aimed to ascertain whether or not condensation was responsible—perhaps in part—for the disappearance of glucose.

Sugar Determination.

	ORIGINAL SOLUTION USED	NH ₄ CNS	NH ₄ CNS PER CC.	GLUCOSE	LOSS
	cc.	cc.	cc.	per cent	per cent
Before	1	22.5	22.5	8.05	
After 36 hrs.	1	21.2	21.2	7.58	5.82

Oxidation.

	ORIGINAL SOLUTION USED	$\frac{N}{5}$ KMnO ₄	ADDITIONAL $\frac{N}{5}$ KMnO ₄	$\frac{N}{5}$ OXALIC ACID	DIFFERENCE	SUGAR	LOSS
	cc.	cc.	cc.	cc.	cc.	per cent	per cent
Before	0.5	40	9.15	22.70	26.45	7.92	
After 36 hrs.	0.5	40	6.37	20.00	26.39	7.90	0.02

Sugar Reduction.

	ORIGINAL SOLUTION USED	NH ₄ CNS	NH ₄ CNS PER CC.	GLUCOSE	LOSS
	cc.	cc.	cc.	per cent	per cent
Before	2	26.00	13.00	4.65	
After 36 hrs.	2	23.40	11.70	4.18	10.10

Oxidation.

	ORIGINAL SOLUTION USED	$\frac{N}{5}$ KMnO ₄	ADDITIONAL $\frac{N}{10}$ KMnO ₄	TOTAL $\frac{N}{5}$ KMnO ₄	$\frac{N}{5}$ OXALIC ACID	$\frac{N}{5}$ KMnO ₄ UTILIZED	GLUCOSE
	cc.	cc.	cc.	cc.	cc.	cc.	per cent
Before	0.5	45.00	4.20	47.10	32.00	15.10	4.50
After 36 hrs.	0.5	45.00	0.60	45.30	30.50	14.80	4.46

Sugar Reduction.

	ORIGINAL SOLUTION USED	NH ₄ CNS	NH ₄ CNS PER CC.	GLUCOSE	LOSS
	cc.	cc.	cc.	per cent	per cent
Before	2	26.10	13.05	4.67	
After 36 hrs.	2	22.80	11.40	4.07	15.00
Hydrolyzed	2	23.20	11.60	4.15	

Oxidation.

	ORIGINAL SOLUTION USED	$\frac{N}{5}$ KMnO_4	ADDITIONAL* $\frac{N}{5}$ KMnO_4	$\frac{N}{5}$ OXALIC ACID	DIFFER- ENCE	SUGAR
	cc.	cc.	cc.	cc.	cc.	per cent
Before	I	40.00	4.70	13.70	31.00	4.65
After 36 hrs. . . .	I	40.00	2.60	9.50	31.10	4.68

* $\frac{N}{10}$ permanganate solution was used in titration against $\frac{N}{5}$ oxalic acid. In this and in the subsequent tables the values are all recalculated as $\frac{N}{5}$ permanganate solution.

Oxidation of Lactic Acid.

For these experiments there was prepared an approximately $\frac{N}{5}$ solution of lactic acid. The lactic acid content of the solution was determined according to F. Ulzer and H. Seidel.⁴ To 10 cc. of the solution an excess of $\frac{N}{10}$ sodium hydroxide solution was added using phenolphthalein as indicator. The mixture was brought to a boil and titrated back with $\frac{N}{10}$ hydrochloric acid solution. It was found that 1 cc. of the lactic acid solution contained 0.016 gram lactic acid. For the experiments the original solution of lactic acid was diluted with nine volumes of water. Two cubic centimeters of the diluted solution were employed for each oxidation experiment. The solution was boiled with 40 cc. of $\frac{N}{5}$ potassium permanganate and 60 cc. of 10 per cent. potassium hydroxide. For decoloration 22.2 cc. of $\frac{N}{5}$ oxalic acid were used. In order to oxidize the excess of oxalic acid it was required to add 37 cc. of $\frac{N}{5}$ potassium permanganate solution. Hence in order to oxidize 20 cc. of lactic acid solution there were employed 21.5 cc. of $\frac{N}{5}$ potassium permanganate. As 1 cc. of $\frac{N}{5}$ potassium permanganate oxidizes 1.5 mgm. lactic acid, the 2 cc. of the original solution oxidized contained 0.0322 gram of lactic acid. The value obtained by titration was 0.0320 gram.

Action of Leucocytes on Lactic Acid.

A leucocyte suspension from 700 cc. of exudate was mixed with Henderson's phosphate solution containing 0.470 gram of lactic acid, as determined by titration. One-half of the lactic acid mixture was analyzed immediately for lactic acid, the other portion

⁴ *Monatsh. d. Chem.*, xviii, pp. 130-141.

after remaining at 37° for thirty-six hours. For this purpose the mixture was freed from protein by boiling and acetic acid. The clear filtrate was neutralized and evaporated to a small volume, acidified with phosphoric acid, ground up with anhydrous sodium sulphate, and extracted with ether. The zinc salt was prepared as already described.

	Zinc Lactate.	Lactic Acid.
By titration	—	0.2350
Control before	0.3158	0.2322
After 36 hours	0.3122	0.2312

Extraction of Lactic Acid.

There were prepared two solutions of lactic acid. One contained glucose, the other sucrose. The concentration of the sugar was 5 per cent. Five grams of glucose or sucrose were dissolved in 100 cc. of water containing a definite amount of lactic acid, as determined on a separate sample by titration or by conversion to the zinc salt. The mixtures of sugar and lactic acid were evaporated to a small volume faintly acidified with phosphoric acid, ground with anhydrous sodium sulphate and extracted with anhydrous ether until portions of the extract failed to give the Uffelmann test. 0.2751 gram zinc lactate was obtained.

Leucocytes and sugar mixtures containing no lactic acid, prepared according to the manner previously described, were subjected to the same treatment. The table summarizes the results.

EXPERIMENT	GLUCOSE	LOSS	LACTIC ACID ADDED	Zn LACTATE RECOVERED	LACTIC ACID	LACTIC ACID REGAINED
	<i>per cent</i>	<i>per cent</i>	<i>gram</i>	<i>gram</i>	<i>gram</i>	<i>per cent</i>
Leucocytes + glucose + lactic acid }	5.01	10		0.2544	0.1815	36.40
Glucose.....{	5.00		0.4725	0.2751	0.1963	41.50
	5.00		0.5000	0.3375	0.2680	53.40
Sucrose.....{	5.00		0.4220	0.5245	0.3750	89.00

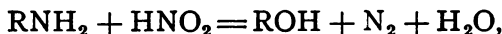
THE QUANTITATIVE DETERMINATION OF ALIPHATIC AMINO GROUPS. II.*

By DONALD D. VAN SLYKE.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATE I.

The author's method for determining aliphatic amino nitrogen¹ by measurement of the nitrogen gas evolved in the reaction,



has proven capable of sufficiently general application² to make it

* Received for publication, July 2, 1912.

¹ This *Journal*, ix, p. 185, 1911.

² It has been utilized in the following articles: Levene and Jacobs: Hefenucleinsäure. III, *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3150; Sjollesma and Rinkes: Hydrolyse des Kartoffeleiweisses, *Zeitschr. f. physiol. Chem.*, lxxvi, p. 506; Kossel and Cameron: Die freien Amidogruppen der einfachsten Proteine, *ibid.*, lxxvi, p. 457; Brach and von Fürth: Chemische Aufbau des Chitins, *Biochem. Zeitschr.*, xxxviii, p. 468; Medigreeanu: Composition of Urinary Albumin, *Journ. of Exper. Med.*, xiv, p. 298; White: Comparative Proteolysis Experiments with Trypsin, *Journ. Amer. Chem. Soc.*, xxxiii, p. 1911; Robinson: Organic Nitrogenous Compounds in Peat Soil, *Tech. Bull. No. 7, Michigan Agricultural Experiment Station*; Mays: Proteinkörper des Liebig'schen Fleisch-extraktes, *Zeitschr. f. physiol. Chem.*, lxxviii, p. 47; Abderhalden and Van Slyke: Bestimmung des Aminostickstoffs in einigen Polypeptiden, *ibid.*, lxxvi, p. 506; Abderhalden and Kramm: Abbau der Proteine im Darmkanal, *ibid.*, lxxvii, p. 425; Abderhalden and Kramm: Abbau der Milcheiweisskörper durch Magensaft unter verschiedenen Bedingungen, *ibid.*, lxxvii, p. 463; Abderhalden and Hanslian: Verwendbarkeit der Estermethode zum Nachweis von Monaminosäuren neben Polypeptiden, *ibid.*, lxxvii, p. 285; Abderhalden: Fütterungsversuche mit vollständig abgebauten Nahrungsstoffen, *ibid.*, lxxvii, p. 22; Osborne and Guest: Hydrolysis of Casein, this *Journal*, ix, p. 333; Osborne and Guest: Hydrolysis of Wheat Gliadin, *ibid.*, ix, p. 425; Levene, Van Slyke and Birchard: Partial Hydrolysis of Proteins. II and III, *ibid.*, viii, p. 269; x, p. 57; Van Slyke and White: Digestion of Protein in the Stomach and Intestine of the Dogfish, *ibid.*, ix, p. 209; Van Slyke: Analysis of Proteins by Determinations of Chemical Groups, *ibid.*, x, p. 15; Van Slyke and Birchard: Nature of the Free Amino Groups in the Native Proteins, *Proc. Soc. Exper. Biol. and Med.*, ix, 1912.

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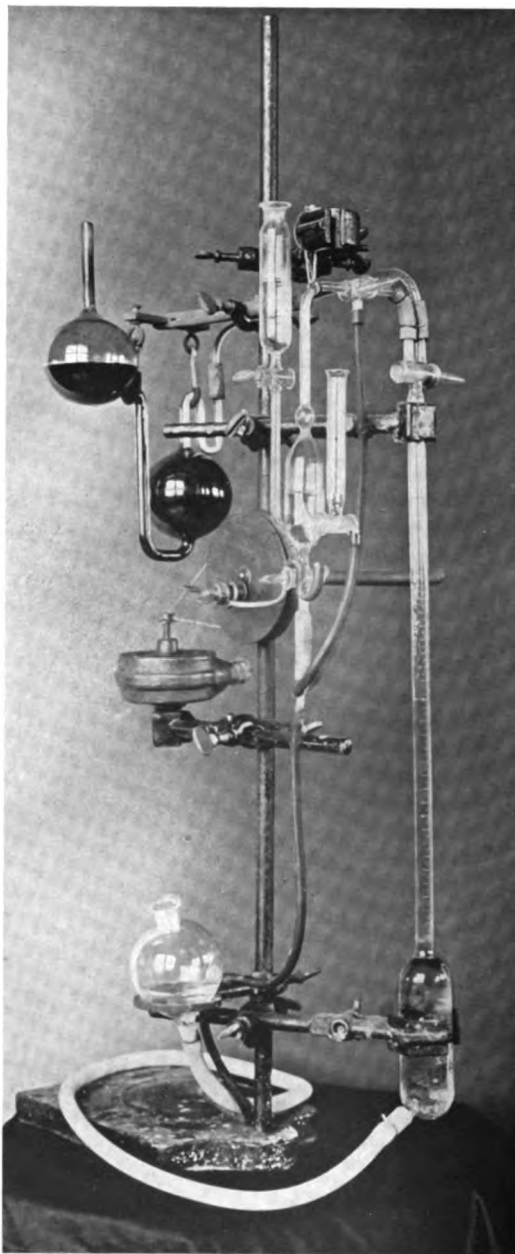


FIG. 1. ENTIRE APPARATUS ASSEMBLED FOR PERMANENT USE.

appear worth while to publish certain improvements made in the apparatus during the past year. Without complicating the manipulation, increasing the cost³ or sacrificing any of the accuracy of the original, the present apparatus has several advantages over the former: (1) The deaminizing vessel, *D*, need not be disconnected from the gas burette between analyses. The apparatus can be used for an indefinite number of determinations without disconnecting the parts. (2) Both the deaminizing bulb *D* and the Hempel pipette can be shaken by a motor. This is a tremendous convenience when considerable series of analyses have to be made.⁴ (3) With the new apparatus one can work even more rapidly than with the original. *D* can be shaken more efficiently with a motor than is possible by hand and the reaction is correspondingly accelerated. Furthermore, during the last stage of the analysis, while the nitric oxide is being removed in the modified Hempel pipette, *D* may be charged with fresh nitrous acid and freed from air (first stage of next analysis), so that one can run off determinations at the rate of one every seven or eight minutes. (4) The motor increases not only the convenience and speed of the method but also its reliability. The only errors likely to occur in the original method were those due to insufficient shaking of *D* or of the pipette; in the former case the nitrogen is incompletely expelled from the nitrous acid solution, and results are too low; in the latter case the nitric oxide is incompletely absorbed and results are too high. The possibility of the occurrence of either error is reduced to a minimum by a mechanical shaking device. (5) The use of rubber tubes to carry off overflows and used up solutions decidedly enhances the neatness of the apparatus, facilitates cleaning, and protects the hands of the manipulator from nitrous acid.

The first of the above cited advantages, freedom from the neces-

³ Robert Goetze, 4 Hörstelstrasse, Leipsic, supplies the glass parts for about Mk. 28; Emil Greiner, 45 Cliff Street, New York, for \$10.

⁴ When the method is used for infrequent analyses, the motor is, of course, superfluous. Even when the shaking is done by hand, however, the new apparatus is more convenient than the old. The parts, minus the driving mechanism, are arranged as shown in the photograph, except that the modified Hempel pipette is brought around to the side of the gas burette opposite the deaminizing bulb where it can be reached more conveniently.

sity of disconnecting the apparatus between analyses, was introduced by Klein⁵ who substituted for the rubber-stoppered deaminizing bottle of the original apparatus, a bulb, to the bottom of which, as in *D*, the inlets and outlets for liquid were sealed, while the gas escaped through a capillary at the top. Klein's modification, compared with the original, had the disadvantages that the amino solutions could not be measured directly into the deaminizing chamber from a burette, like *B*, but had to be washed in, and that the time required for the determination was lengthened by the changes required in the manipulation. Nevertheless, the advantage of a one-piece, all-glass deaminizing vessel was made so evident by the use of Klein's modification that we attempted to combine this advantage with those of our original apparatus. The present apparatus is the result of this attempt, with the additional adaptation of both deaminizing vessel and Hempel pipette to use with a mechanical shaker.

The structure of the apparatus and the manner in which it is set up are apparent from the accompanying cut and photograph.

D is of 40-45 cc. capacity, *A* of about 35 cc. and the burette *B* of 10 cc. The wire from which the deaminizing bulb *D* is suspended should be fairly stiff, and rigidly fastened in position from above so that the loop about the capillary acts as a fixed center. *A* is then so placed that its center of gravity comes near this center and the shaking of *D* is accomplished with a minimum motion in *A* and, consequently, without putting a dangerous strain on the tube which connects *A* with *D*. This tube is strong-walled and of 3 mm. inner diameter. It is essential that the bore of cock *a* should also be 3 mm. The reason for this is that during the analysis gas containing some nitrogen collects in the tube. Unless *a* is of as wide bore as the tube the liquid from *A* may flow around the bubble instead of forcing it into *D* at the end of the reaction. The cock *d* is also of large bore in order to facilitate emptying *D*. The neck connecting *D* and *B* must be of at least 8 mm. inner diameter in order to allow free circulation of the solution in *D* up to the cock *B*. The small bulb at the top of *D* keeps the reacting solution from splashing into the capillary.

In order to insure tightness of the cocks and to prevent their becoming loosened by vigorous shaking it is well to lubricate them with a paste made by dissolving together over a flame one part of rubber, one part of paraffin and two parts of vaseline.

The structure of the modified Hempel pipette is entirely apparent from the photograph. This form would undoubtedly facilitate absorption in all gas analyses where shaking is necessary.

The driving wheel, as can be seen from the photograph, is so arranged that

⁵ This *Journal*, x, p. 287.

it can be used alternately to shake the deaminizing bulb or the Hempel pipette. The driving rod is shown in position for shaking the deaminizing bulb. By lifting the rod from the shoulder of *D* and placing the other hook, at the end of the rod, over the horizontal lower tube of the pipette, the power is transferred to

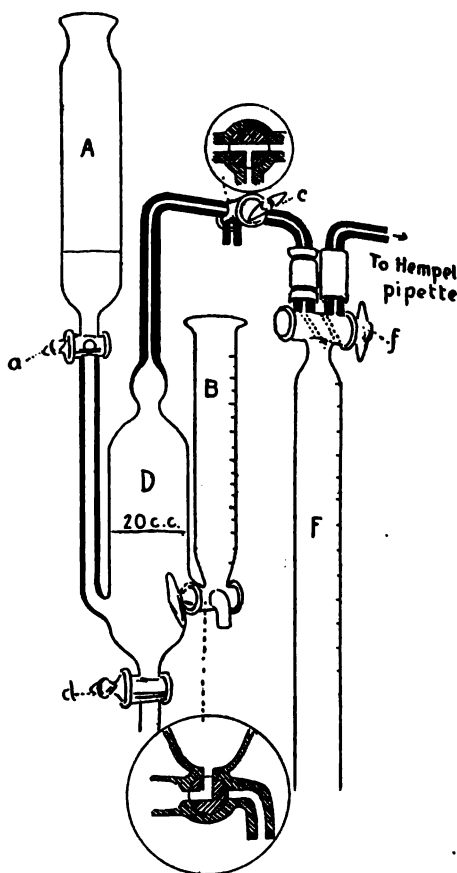


FIG. 2. DEAMINIZING BULB AND CONNECTIONS IN DETAIL.

the latter. Rubber tubes drawn over the hooks at the end of the driving rod and those from which the Hempel pipette is suspended make the apparatus almost noiseless. For power one can use a good water motor. Still more convenient is a small electric motor, particularly when connected with a rheostat enabling one to regulate the speed. The gearing should be so arranged that the driving wheel, to which the driving rod is eccentrically attached, makes 300 to 500 revolutions per minute. The driving rod is attached about 1.5 cm., in no case over 2 cm., from the center of the wheel.

The manipulation is in principle the same as described in this *Journal*, ix, pp. 189-91. As there are slight variations due to the different form of the present apparatus, however, we describe the present technique, dividing the determination into three stages, as in the original description.

1. *Displacement of Air by Nitric Oxide.*—Water from *F* fills the capillary leading to the Hempel pipette and also the other capillary as far as *c*. Into *A* one pours a volume of glacial acetic acid sufficient to fill one-fifth of *D*. For convenience, *A* is etched with a mark to measure this amount. The acid is run into *D*, cock *c* being turned so as to let the air escape from *D*. Through *A* one now pours sodium nitrite solution (30 gm. NaNO_2 to 100 cc. H_2O) until *D* is full of solution and enough excess is present to rise a little above the cock into *A*. It is convenient to mark *A* for measuring off this amount also. The gas exit from *D* is now closed at *c*, and, *a* being open, *D* is shaken for a few seconds. The nitric oxide, which instantly collects, is let out at *c*, and the shaking repeated. The second crop of nitric oxide, which washes out the last portions of air, is also let out at *c*. *D* is now connected with the motor and shaken till all but 20 cc. of the solution have been displaced by nitric oxide and driven back into *A*. A mark on *D* indicates the 20 cc. point. One then closes *a* and turns *c* and *f* so that *D* and *F* are connected. The above manipulations require between one and two minutes.

2. *Decomposition of the Amino Substance.*—Of the amino solution to be analyzed 10 cc. or less, as the case may be, are measured off in *B*. Any excess added above the mark can be run off through the outflow tube. The desired amount is then run into *D*, which is already connected with the motor, as shown in the photograph. It is shaken, when α -amino-acids are being analyzed, for a period of three to five minutes. With α -amino-acids, proteins or partially or completely hydrolyzed proteins, we find that at the most five minutes' vigorous shaking completes the reaction.* Only in the cases of some native proteins which, when deaminized, form un-

* Only 95 per cent of the lysine nitrogen reacts in five minutes, but the remaining one-twentieth of the lysine nitrogen is a practically negligible proportion of the total nitrogen of a complete protein.

wildly coagula that mechanically interfere with the thorough agitation of the mixture, a longer time may be required. In case a viscous solution is being analyzed and the liquid threatens to foam over into *F*, *B* is rinsed out and a little caprylic alcohol is added through it. For amino substances, such as amino-purines, requiring a longer time than five minutes to react (cf. p. 191, former article), one merely mixes the reacting solutions and lets them stand the required length of time, then shakes about two minutes to drive the nitrogen completely out of solution.

When it is known that the solution to be analyzed is likely to foam violently, it is advisable to add caprylic alcohol through *B* before the amino solution. *B* is then rinsed with alcohol and dried with ether or a roll of filter paper before it receives the amino solution.

3. *Absorption of Nitric Oxide and Measurement of Nitrogen.*—The reaction being completed, all the gas in *D* is displaced into *F* by liquid from *A* and the mixture of nitrogen and nitric oxide is driven from *F* into the absorption pipette. The driving rod is then connected with the pipette by lifting the hook from the shoulder of *d* and placing the other hook, on the opposite side of the driving rod, over the horizontal lower tube of the pipette. The latter is then shaken by the motor for a minute, which, with any but almost completely exhausted permanganate solutions, completes the absorption of nitric oxide. The pure nitrogen is then measured in *F*. During the above operations *a* is left open, to permit displacement of liquid from *D* as nitric oxide forms in *D*.

Testing Completeness of Reaction.—Particularly when the mechanical shaker is used, there is little danger of failing to obtain a complete evolution of nitrogen. The point may be tested, however, as follows. The nitrogen from *F* is driven out at *c*; *a* is closed and *D* connected with *F*. The gas which has formed in the nitrous acid solution in *D* during the absorption of the nitric oxide and measurement of nitrogen is shaken out and driven over into *F* and then into the Hempel pipette as before. After absorption of the nitric oxide, the gas left should not measure more than that obtained in blank tests, usually less than 0.1 cc. After the gas has all been forced from *D* over into *F* at the end of the reaction, the

nitrous solution is run out from *D*, by opening *d*, through a tube leading to a drain. *B* is rinsed and dried with a roll of filter paper or with alcohol and ether and the apparatus is immediately ready for use again.

Blank determinations, performed as above except that 10 cc. of distilled water replaces the solution of amino substance, must be performed on every fresh lot of nitrite used. The amount of gas obtained on a five-minute blank is usually 0.3 to 0.4 cc. with very little increase for longer tests. Nitrite giving a much larger correction should be rejected.

The following determinations, performed with an N/10 solution of leucine, indicate the speed of the reaction. The correction applied for reagents was 0.40 cc. Ten cubic centimeters of N/10 leucine solution, containing 14.01 mgm. of nitrogen, were used for each determination.

TIME OF REACTION	N	TEMPERATURE	PRESSURE	N OBTAINED	N OBTAINED ON SECOND SHAKING OF SOLUTION	TOTAL N OBTAINED
<i>minutes</i>	<i>cc.</i>	<i>degrees C.</i>	<i>mm.</i>	<i>mgm.</i>	<i>cc.</i>	<i>mgm.</i>
2	24.38	23	762	13.71	0.45	13.97
3	24.65	22	762	13.93	0.20	14.03
4	24.80	22	762	14.01	0.00	14.01
10	25.07	24	762	14.03	0.00	14.03

The driving wheel was making 300 revolutions per minute. At speeds of 400 or 500 revolutions the reaction can be driven to completion in three, or, with higher room temperature, in two minutes.

The rate of reaction of *ammonia* is shown in the following table. Ten cc. portions of N/5 ammonium sulphate solution, containing 28.02 mgm. of nitrogen each, were used.

TIME OF REACTION	N	TEMPERATURE	PRESSURE	WEIGHT OF N	PER CENT OF TOTAL AMMONIA NITROGEN
<i>minutes</i>	<i>cc.</i>	<i>degrees C.</i>	<i>mm.</i>	<i>mgm.</i>	
3	12.1	24	752	6.86	21.6
5	18.4	24	752	10.16	36.3
10	31.5	24	752	17.38	62.1

As pointed out before, ammonia reacts slowly compared with the amino-acids. *For accurate determination of NH₂ nitrogen in digesting solutions, etc., it is advisable to first remove the ammonia;*

although good comparative results can be obtained, in the presence of the relatively small proportion of ammonia usually present, if reaction, conditions of time, temperature, and concentration of solutions are kept constant, so that the proportion of the ammonia decomposed is the same in each determination. The ammonia can be conveniently removed and determined by distillation with $\text{Ca}(\text{OH})_2$ under diminished pressure, as described on page 21, vol. X of this *Journal*. After the distillation the excess $\text{Ca}(\text{OH})_2$ is dissolved with acetic acid. It is essential that all the ethyl alcohol should be distilled off, as it decomposes nitrous acid with formation of large volumes of gases which can be removed with permanganate only with difficulty and by the use of perfectly fresh permanganate solution. The point at which the alcohol has all been boiled off is usually indicated when the solution begins to foam in the distilling flask.

The following results were obtained with lysine picrate. Lysine, as previously stated, reacts more slowly than the other amino-acids because it contains not only an α -amino group but also an ω -amino group. In the fifteen and thirty-minute determinations the solution was shaken only during the last five minutes.

WEIGHT OF LYSINE PICRATE	TIME OF REACTION	N	TEMPERATURE	PRESSURE	$\text{NH}_4\text{-N}$ FOUND	$\text{NH}_4\text{-N}$ CALCULATED
<i>gram</i>	<i>minutes</i>	<i>cc.</i>	<i>degrees C.</i>	<i>mm.</i>	<i>per cent</i>	<i>per cent</i>
0.200	5	25.4	24	764	7.13	7.47
0.200	15	26.7	24	764	7.49	7.47
0.200	30	26.7	24	764	7.49	7.47

Solutions to be analyzed should be free of ethyl alcohol and acetone. These substances when mixed with nitrous acid give off gases or vapors which are with difficulty absorbed by the permanganate.

Amyl alcohol, which in the original description of the amino method was recommended to prevent the foaming of viscous solution, must be replaced for this purpose by caprylic alcohol [Kahlbaum's "octyl-alkohol (sekundär) I"]. Amyl alcohol, boiling at 131°, has the disadvantage of a very noticeable vapor tension. Permanganate solution apparently possesses the power to absorb slight amounts of amyl alcohol vapor. Particularly on hot days, however,

and when relatively much of the alcohol is used, it is necessary to change the permanganate with every analysis or else reduce the volume of gas observed by multiplication with an empirically determined factor.

The following determinations illustrate this point. They were performed with N/10 leucine⁷ solution, using 1 cc. of amyl alcohol in each case. The temperature was 27°, the pressure 756 mm. The analyses were done in rapid succession, using the same permanganate solution with all.

NUMBER	N 10 LEUCINE SOLUTION	GAS OBSERVED	GAS CALCULATED FOR N	GAS OBSERVED GAS CALCULATED
	cc.	cc.	cc.	
1	10	26.1	25.7	1.015
2	10	27.8	25.7	1.082
3	10	27.6	25.7	1.079
4	10	27.6	25.7	1.079
5	5	13.8	13.05	1.079

Theoretically a higher alcohol should be more adapted to the purpose, since, as the size of the alcohol molecule increases, the volatility diminishes, while the effectiveness in reducing the surface tension of aqueous solutions increases. Kahlbaum's secondary octyl (caprylic) alcohol, which is not very expensive, was found satisfactory in every respect. It is so efficient in preventing foam that, when a few drops are added, a 2 or 3 per cent egg albumin solution can be analyzed without difficulty, even when the reacting solution is shaken rapidly with a motor. The fact that it does not interfere to the slightest extent with the accuracy of the results is shown by the following determinations. They were performed

NUMBER	N 10 LEUCINE SOLUTION	GAS OBSERVED	GAS CALCULATED FOR N
	cc.	cc.	cc.
1	10.00 ± 0.04	25.95	25.95 ± 0.10
2	10.00 ± 0.04	25.90	25.95 ± 0.10
3	10.00 ± 0.04	25.95	25.95 ± 0.10
4	10.00 ± 0.04	26.00	25.95 ± 0.10
5	10.00 ± 0.04	25.85	25.95 ± 0.10

in the same manner as those with amyl alcohol and with the same

⁷For results with glycocoll, cf. accompanying paper on glycocoll picrate.

ure.

<i>l</i>	728	730	730	762	764	766	768	770	772	<i>l</i>
11°	0.5680	0.5695	0.5935	0.5950	0.5965	0.5980	0.5995	0.6010	0.6030	11°
12°	0.5655	0.5670	0.5905	0.5925	0.5940	0.5955	0.5970	0.5985	0.6000	12°
13°	0.5630	0.5645	0.5880	0.5895	0.5910	0.5930	0.5945	0.5960	0.5975	13°
14°	0.5605	0.5620	0.5855	0.5870	0.5885	0.5900	0.5915	0.5935	0.5950	14°
15°	0.5580	0.5595	0.5830	0.5845	0.5860	0.5875	0.5890	0.5905	0.5920	15°
16°	0.5555	0.5570	0.5800	0.5815	0.5830	0.5850	0.5865	0.5880	0.5895	16°
17°	0.5525	0.5540	0.5775	0.5790	0.5805	0.5820	0.5825	0.5850	0.5865	17°
18°	0.5500	0.5515	0.5745	0.5765	0.5780	0.5795	0.5810	0.5825	0.5840	18°
19°	0.5475	0.5490	0.5720	0.5735	0.5750	0.5765	0.5780	0.5795	0.5810	19°
20°	0.5445	0.5460	0.5690	0.5705	0.5725	0.5740	0.5755	0.5770	0.5785	20°
21°	0.5420	0.5435	0.5665	0.5680	0.5695	0.5710	0.5725	0.5740	0.5755	21°
22°	0.5395	0.5410	0.5635	0.5650	0.5665	0.5680	0.5695	0.5715	0.5730	22°
23°	0.5365	0.5380	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5700	23°
24°	0.5335	0.5350	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	24°
25°	0.5310	0.5325	0.5550	0.5565	0.5580	0.5595	0.5610	0.5625	0.5640	25°
26°	0.5260	0.5295	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	26°
27°	0.5250	0.5265	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	27°
28°	0.5220	0.5235	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	28°
29°	0.5195	0.5210	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	29°
30°	0.5160	0.5175	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	30°
<i>l</i>	728	730	730	762	764	766	768	770	772	<i>l</i>

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N/10 leucine solution. The temperature was 29°, as warm as a laboratory often becomes, and the pressure 756 mm. The first determination was a control, without the octyl alcohol.

For convenience in calculating results the following table is appended. The figures are calculated by dividing by 2 those for moist nitrogen given by Gattermann in the *Praxis des organischen Chemikers*, ninth edition. They represent the weights of amino nitrogen in milligrams which correspond to 1 cc. of nitrogen gas, obtained by the action of nitrous acid and measured over water, at the temperatures and atmospheric pressures indicated.

THE COMPOSITION AND PROPERTIES OF GLYCOCOLL PICRATE AND THE SEPARATION OF GLYCOCOLL FROM ALANINE.*

BY P. A. LEVENE AND DONALD D. VAN SLYKE.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Glycocoll picrate was first described by Levene,¹ who demonstrated its value in separating the mixture of glycocoll and alanine encountered in the hydrolysis of most proteins. He assigned to it the structure, $C_2H_5NO_2.C_6H_3N_3O_7$, regarding the substance as a combination of one molecule each of glycocoll and picric acid. We find that this was an error, and that, as reported by us some months ago at the Christmas meeting of the Society of Biological Chemists, the picrate contains *two* molecules of glycocoll in combination with one of picric acid. The fact that Levene and others who have since used the picrate in the isolation of glycocoll have not detected this error is easy to explain. Picric acid and glycocoll contain practically the same percentage of carbon and nitrogen, so that determinations of the percentages of these elements, which are ordinarily most to be relied upon, give no clue whatever to the proportions in which the two constituents of the salt are present. Even the difference in hydrogen contents is so low that picrates with respectively one and two molecules of glycocoll would vary by only 0.8 in their hydrogen percentage. Consequently the entire elementary composition affords no reliable data for determination of the structure of the substance.

In addition to this, the problem was complicated by the readiness with which free picric acid crystallizes. When enough or more than enough picric acid to form the salt of supposed monomolecular composition is added in hot solution to the glycocoll, a portion of the excess of picric acid crystallizes with the picrate when the

* Received for publication, July 2, 1912.

¹ This *Journal*, i, p. 413.

solution is cooled. In this manner it is easy to obtain mixtures of glycocoll picrate and free picric acid containing almost as much of the latter as is required by the monomolecular formula. The excess picric acid is, however, merely a mechanical admixture. It can be removed by recrystallizing the substance, or by merely shaking it out with ether, which leaves behind the pure salt, $(C_2H_5NO_2)_2.C_6H_3O_7N_3$.

For determining analytically the purity of the salt, and in particular its freedom from excess picric acid, the elementary composition is, as already stated, of little significance. Two other methods are available: (1) The proportion of glycocoll can be estimated by determination of the amino nitrogen by the nitrous acid method.² This is the simplest method, and the one by which the correct composition of the picrate was first detected. (2) The salt can be decomposed with an excess of mineral acid, the picric acid shaken out with ether and the glycocoll and picric acid weighed separately. Results by this method confirm those by the amino determination.

EXPERIMENTAL.

The Reaction of Glycocoll with Nitrous Acid.

As already stated by one of us,³ glycocoll when treated with nitrous acid gives off a volume of gas, not absorbed by alkaline permanganate, which exceeds by several per cent that calculated for one molecule of nitrogen. Results are sufficiently constant, however, to enable one to obtain good figures for the amino nitrogen if an empirical correction is used. As the amino determination is the only practical method for analysis of the picrate, the following analyses of glycocoll were made in order to determine accurately the empirical correction under definite conditions.

In the analyses given below the solutions of glycocoll were mixed with the nitrous acid in the apparatus described in the previous article. The reacting solutions were allowed to stand five, eleven or thirty minutes, then shaken one minute by hand to expel the dissolved nitrogen. Glycocoll contains 18.69 per cent of nitrogen.

² Van Slyke: this *Journal*, ix, p. 185; also, cf. preceding article.

³ Van Slyke: this *Journal*, ix, p. 199.

WEIGHT OF GLYCOCOLL	TIME OF REACTION	GAS MEASURED	TEMPERATURE	PRESSURE	PER CENT N CALCULATED FROM GAS VOLUME	PER CENT OF THEORETICAL N
<i>gram</i>	<i>minutes</i>	<i>cc.</i>	<i>degrees C.</i>	<i>mm.</i>		
0.0801	6	27.8	21	770	19.94	106.7
0.0801	12	28.7	21	770	20.59	110.1
0.0801	31	29.2	21	770	21.01	112.4

The following results were obtained from analyses in which the reacting solutions were shaken constantly and rapidly with a motor from the time at which they were mixed till the reaction was discontinued.

WEIGHT OF GLYCOCOLL	TIME OF REACTION	GAS MEASURED	TEMPERATURE	PRESSURE	PER CENT N CALCULATED FROM GAS VOLUME	PER CENT OF THEORETICAL N
<i>gram</i>	<i>minutes</i>	<i>cc.</i>	<i>degrees C.</i>	<i>mm.</i>		
0.0768	1	28.0	24	754	19.72	105.5
0.0768	2	28.6	24	754	20.13	107.7
0.0768	3	28.7	24	754	20.20	108.0
0.0768	5	28.6	24	754	20.13	107.7
0.0768	5	28.25	20	758	20.12	107.7
0.0768	40*	29.00	20	758	20.58	111.3

* The 40-minute determination was shaken only during the last five minutes.

From the above figures it is apparent that when the reacting solutions are allowed to stand five minutes and are then shaken one minute, the results require, for correction, multiplication by the factor, $\frac{100}{106.7}$ or 0.94, while, when the mechanical shaker is used for two to five minutes, a factor of 0.93 is required. The above conditions and corrections were used in the analyses of glycocoll picrate, with consistent results. It should be noted, that while making analyses of pure glycocoll solutions one must change the permanganate absorbent solution oftener than when analyzing other amino-acids, as the abnormal gases given off by glycocoll are less completely taken up by the permanganate after the latter has been used for four or five analyses of this amino-acid.

Composition of Glycocoll Picrate.

1. Samples of glycocoll picrate prepared according to Levene's original directions, using 4 parts of picric acid to 1 of glycocoll (1.5 parts are the proportion actually required to form $(C_2H_5NO_2)_2.C_6H_3N_3O_7$), showed amino nitrogen contents varying

between that required for the supposed monoglycine picrate and diglycine picrate. The melting points varied between 185° and 195° . When such picrates were recrystallized from enough solvent to hold in solution the free picric acid present the pure diglycine picrate was obtained, softening at about 200° and decomposing at 202° . The following case is an example. A picrate obtained by Levene's original method from gelatin showed an amino content of 6.9 per cent. and a melting point of 188° . It was recrystallized from water and then melted at 200° . It gave the following figures on analysis:

Substance, 0.1900; 25.7 cc. N at 768 mm., 21° (with HNO_3).

Substance, 0.1541; CO_2 , 0.1796; H_2O , 0.0492.

	Calculated for $(\text{C}_6\text{H}_5\text{NO}_3)_3 \cdot \text{C}_6\text{H}_5\text{N}_2\text{O}_7$	Found.
$\text{NH}_2\text{-N}$	7.39	7.28
C	31.67	31.76
H	3.43	3.57

Five grams of the picrate were dissolved in 50 cc. of hot water and the picric acid freed with 30 cc. of $\text{N}/1$ sulphuric acid. The picric acid was shaken out with ether. Evaporation of the ethereal extract yielded 3.001 grams of picric acid melting at 122° . The sulphuric acid in the aqueous solution was precipitated by the addition of an exact equivalent of barium hydrate solution which had been titrated against the same $\text{N}/1$ sulphuric acid. The filtrate from the barium sulphate was concentrated to dryness and yielded 2.007 grams of glycocoll melting at $228\text{--}230^{\circ}$.

	Calculated for $(\text{C}_6\text{H}_5\text{NO}_3)_3 \cdot \text{C}_6\text{H}_5\text{H}_2\text{O}_7$	Found.
Picric acid	60.26	60.02
Glycocoll	39.74	40.15

The glycocoll gave the following figures on analysis:

Substance, 0.1240; CO_2 , 0.1463; H_2O , 0.0752.

	Calculated for $\text{C}_2\text{H}_5\text{NO}_2$	Found.
C	31.97	32.19
H	6.73	6.84

2. Samples of glycine picrate can readily be prepared, containing abnormally high amounts of picric acid, by crystallization from solutions containing more than the required 0.5 molecule of picric acid per molecule of glycocoll. That the excess of picric acid is merely a mechanical admixture, however, can be shown by extracting it with anhydrous ether.

Two grams of glycocoll and 6 grams (1 molecule) of picric acid were dissolved in 20 cc. of hot water and allowed to crystallize at 0°. The crystals were washed with ice water, followed by a little alcohol and ether and dried in a vacuum. Yield, 3.79 grams. The substance softened at 185° and melted at 190°.

Analysis.—0.1726 gram substance; 18.7 cc. N at 20°, 764 mm. Using the factor 0.94, this gives amino nitrogen as 5.85 per cent. The calculated amount for the diglycine picrate is 7.39, for the monoglycine picrate, 4.61 per cent.

The substance was ground fine and extracted with several portions of boiling anhydrous ether, the extraction being continued until the extracts were no longer colored. The residue now obtained melted at 200° and the amino nitrogen content was raised to nearly the calculated value for the pure diglycine picrate.

Analysis.—0.1529 gram substance; 20.25 cc. N at 18°, 760 mm.

	Calculated for (C ₂ H ₅ NO ₂) ₂ ·C ₆ H ₃ N ₃ O ₇ .	Found.
NH ₂ -N	7.39	7.19
As before, the factor 0.94 was used.		

Another sample with high picric acid content was obtained by dissolving 7.6 grams of diglycine picrate in 20 c.c. of water with 4.6 grams (1 molecule) of picric acid. The product, obtained by crystallization at 0°, weighed 5.49 grams and contained only 5.49 per cent of amino nitrogen. After extraction with ether it gave figures for the pure diglycine picrate.

Analysis.—0.1423 gram substance; 19.5 cc. N at 21°, 770 mm. .

	Calculated for (C ₂ H ₅ NO ₂) ₂ ·C ₆ H ₃ N ₃ O ₇ .	Found.
NH ₂ -N	7.39	7.42

Incidentally these experiments bring out the fact that the presence of an excess of free picric acid greatly increases the solubility of glycine picrate. Twenty cubic centimeters of water at 0° dissolve only 0.35 gram of diglycine picrate, equivalent to 0.14 gram of glycocoll. In the presence of an excess of picric acid, however, there remained in solution in the first of the above two experiments, 0.8 gram of glycocoll, and in the second, 1.4 grams.

3. The presence of free picric acid does not prevent the crystallization of diglycine picrate in pure condition, provided the solvent used is capable of holding in solution all the excess picric acid. The latter decreases the yield of diglycine picrate, but does not affect its composition nor crystallize with it.

In each of the following three experiments 2 grams of Kahlbaum's glycocoll were dissolved in 10 cc. of water. To these solutions were added respectively one, two and three times the amount of picric acid required to form diglycine picrate, the acid being in each case dissolved in 40 cc. of 95 per cent alcohol before it was added to the glycocoll solution. It was previously ascertained that 50 cc. of 76 per cent alcohol are capable of holding in solution, even at 0°, the amount of free picric acid present. The solutions were allowed to stand over night at 0° for crystallization to become complete. The crystals were washed in each case with uniform portions of 50 per cent alcohol, 95 per cent alcohol and once with ether. The results of the experiments are given in the following table.

GLYCOCOLL	PICRIC ACID	YIELD OF GLYCINE PICRATE	AMINO N CONTENT	NH ₂ -N CALCULATED
<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>per cent</i>	<i>per cent</i>
2	3	4.53	7.45	7.39
2	6	2.87	7.44	7.39
2	9	1.52	7.41	7.39

The picrate melted at 199°–200° in each case.

Conditions for Precipitation of Glycine Picrate.

Glycine picrate is much less soluble in alcohol than in water; but, as alcohol precipitates the amino-acids from which glycocoll has usually to be separated, the conditions for precipitation of the latter as completely as possible in water solutions had to be determined. In the following experiments a supersaturated solution was made by dissolving glycine picrate in warm water. Portions of this solution were cooled to the temperatures indicated and maintained there for several hours. They were then filtered and 10 cc. portions of the filtrates used for determinations of amino nitrogen. All the solutions deposited crystals as soon as they were cooled to their respective temperatures and they were stirred occa-

sionally while standing in order to assure approximate attainment of solubility equilibrium.

TEMPERATURE	N	TEMPERATURE OF N	PRESSURE	GRAMS GLYCOCOLL DISSOLVED IN 100 CC.	GRAMS GLYCINE PICRATE DISSOLVED IN 100 CC.
<i>degrees C.</i>	<i>cc.</i>	<i>degrees C.</i>	<i>mm.</i>		
21	29.6	16	754	1.72	4.35
11	16.2	13	752	0.95	2.42
0	11.8	16	766	0.698	1.76
0	11.95	18	764	0.698	1.76

From the above it is evident that the solubility of glycine picrate rises very rapidly with the temperature and that cooling completely to 0° is necessary to attain most complete precipitation.

The following experiment was performed in order to determine approximately how much time is required for precipitation at 0° to reach its maximum. A 10 per cent solution of glycine picrate was cooled to 0° by shaking the containing flask in ice water. Crystallization began before the solution reached 0° and was complete within fifteen minutes after that time. Portions of the solution were filtered off and used for amino determinations as described in the preceding experiment.

TIME OF REACTION	N	TEMPERATURE OF N	PRESSURE	GRAMS GLYCOCOLL DISSOLVED IN 100 CC.	GRAMS GLYCOCOLL PICRATE DISSOLVED IN 100 CC.
	<i>cc.</i>	<i>degrees C.</i>	<i>mm.</i>		
15 min.	11.90	18	764	0.694	1.75
3 hrs.	12.20	22	764	0.698	1.76

Gravimetric solubility determinations showed that 50 per cent alcohol at 21° dissolves per 100 cc., 2.13 grams of glycine picrate, absolute alcohol only 0.16 gram.

Separation of Glycocoll from Alanine.

In a solution containing only glycocoll and picric acid it has been shown above that the presence of an excess of picric acid above the amount required to form diglycine picrate increases the solubility of the latter and makes the precipitation less complete. When, however, alanine is also present it prevents the solvent effect of an excess of picric acid, at least so long as the amount of the latter does not exceed one-half molecule for each atom of amino-acid

nitrogen present. In fact, the presence of excess picric acid up to this point actually depresses, the solubility of the glycine picrate, and makes its precipitation more complete than when only enough picric acid to combine with the glycocoll is added. The following two experiments illustrate this point.

Separation of Alanine and Glycocoll, Using Only Enough Picric Acid to Combine with the Glycocoll.—One gram of glycocoll, 1.5 grams (1 molecule) of picric acid and 1 gram of alanine were dissolved in 15 cc. of hot water and cooled to 0°. After several hours the crystals were filtered on a cold suction funnel, washed twice with a few cubic centimeters of ice water and then several times with 95 per cent alcohol. The dried product weighed 1.96 grams, containing 0.78 gram of the original 1.00 gram of glycocoll. The picrate was pure, softening first at 200° and decomposing at 202°.

Separation of Glycocoll from Alanine, Using One-Half Molecule of Picric Acid for Each Molecule of Amino-Acid.—One gram of glycocoll, 1 gram of alanine and 2.78 grams of picric acid were dissolved in 15 cc. of water. The precipitate was treated in the same manner as that in the preceding experiment. In this case, however, the yield was larger, 2.22 grams of picrate, equivalent to 0.89 gram of glycocoll. The melting point was 197°, showing that the picrate was not entirely pure. This was confirmed by the analysis, which, however, indicates that the picrate was 96 per cent pure, containing about 4 per cent of free picric acid.

Substance, (I) 0.1550 gram, (II) 0.1680 gram; N, (I) 20.3 cc., (II) 22.2 cc. at 20°, 764 mm.

	Calculated for (C ₂ H ₅ NO ₂) ₂ .C ₆ H ₅ N ₃ O ₇ .	I	Found.	II
NH ₂ -N	7.39	7.05		7.11

Separation of Glycocoll from Alanine, with Isolation of the Alanine.—The alanine used in this experiment, like that in the two preceding, was an optically active sample prepared from hydrolyzed silk and purified by repeated recrystallization. It was analytically pure and gave the following figures for rotation in the yellow rays of a light purified by a Schmidt and Haensch spectroscope.

Substance, 0.1214; solution, containing an equivalent of HCl, 1.568 grams; concentration, of alanine hydrochloride, 10.91 per cent; sp. gr., 1.03; rotation in 1 dm. tube, + 1.17°.

$$[\alpha]_D^{20} = + 10.5^\circ \pm 0.1^\circ.$$

Fischer gives $10.4^\circ \pm 0.2^\circ$. The glycocoll used was Kahlbaum's.

One gram of alanine, 1 gram of glycocoll and 1.8 grams of picric acid (0.6 molecule for the glycocoll) were dissolved in 7 cc. of hot water. The solution was allowed to stand at 0° until crystallization was complete. The crystals were washed as before on an ice-cold suction funnel, with a little ice water, followed by alcohol. The yield was 2.29 grams, equivalent to 0.91 gram of glycocoll. The product decomposed at 202° and was quite pure.

Analysis.—0.1611 gram substance; 22.9 cc. N.

	Calculated for (C ₂ H ₅ NO ₂) ₂ ·C ₆ H ₃ N ₃ O ₇ .	Found.
NH ₃ -N	7.39	7.40

The analysis was performed with the mechanical shaker so that the factor 0.93 was used.

The filtrate from the picrate was acidified with 20 cc. of N/1 sulphuric acid and shaken out several times with ether, until the yellow color of picric acid had entirely disappeared. The solution was then heated on the steam bath and the sulphuric acid was precipitated by the addition of an exact equivalent of barium hydrate solution which had previously been titrated against the sulphuric acid used. The filtrate from the barium sulphate was evaporated to dryness. It left 1.09 grams of residue which contained 1.0 per cent of ash. The substance on combustion gave figures approximating those for alanine, but 0.5 per cent low, indicating the presence of a small proportion of glycocoll.

Substance (ash-free), 0.1275 gram; CO₂, 0.1871; H₂O, 0.0890.

	Calculated for C ₂ H ₅ NO ₂ .	Calculated for C ₆ H ₃ NO ₃ .	Found.
C	31.97	40.42	39.92
H	6.73	7.93	7.85

The sharpest differentiation between the two substances is given by the rotation, as glycocoll is inactive. The rotation, like the yields of picrate and alanine, indicated that 9 per cent of the glycocoll had gone into the filtrate with the alanine.

Substance (ash-free), 0.1120 gram; solution, containing an equivalent of HCl, 1.551 grams; concentration, of alanine hydrochloride, 10.15 per cent; sp. gr., 1.03; rotation, $+0.99^{\circ}$.

$$[\alpha]_D^{20} = +9.5^{\circ} \pm 0.1^{\circ}.$$

We confirmed the experience of Levene in failing entirely to obtain a picrate of alanine. No compound of picric acid and alanine could be induced to crystallize from any concentration in water solution; and, when such a solution of alanine and picric acid was precipitated by addition of alcohol, the precipitate consisted of pure alanine.

SUMMARY.

Glycine picrate is composed of two molecular weights of glycocoll in combination with one of picric acid. It softens at 199° – 200° and decomposes at 202° . A correct melting point is ordinarily good proof of its purity, as the presence of a few per cent of free picric acid or of alanine depresses the melting point several degrees. The picrate is extremely soluble in hot water. At 0° , however, 100 cc. dissolve only 1.76 grams of picrate, containing 0.7 gram of glycocoll.

To separate glycocoll from alanine, the mixture is dissolved in three to four parts of hot water. In this solution is also dissolved an amount of picric acid which exceeds that required to combine with the glycocoll present (1.5 grams of picric acid combine with 1 gram of glycocoll), but does not exceed that required to combine with the mixture, if all the amino nitrogen present is calculated as belonging to the glycocoll. The solution is cooled to 0° and allowed to remain at that temperature until crystallization is complete, which usually occurs in less than an hour. The glycine picrate is washed with a slight amount of water at 0° , followed by 95 per cent alcohol. The purity is controlled by the melting point and amino nitrogen determination. The filtrate from the picrate is treated with an excess of $N/1$ sulphuric acid and freed from picric acid with ether. The sulphuric acid is then precipitated by an equivalent of titrated barium hydrate solution. The alanine is left as a residue when the filtrate from the barium sulphate is concentrated to dryness. It still contains several per cent of glycocoll, but can be obtained, as above described, over 90 per cent pure.

THE CONDITIONS FOR COMPLETE HYDROLYSIS OF PROTEINS.*

By DONALD D. VAN SLYKE.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Henriques and Gjaldbaek,¹ in an article devoted chiefly to a study of the above subject with the formol titration, find that, when proteins are hydrolyzed with acid, both amino nitrogen and ammonia increase up to a certain point, at which the amino nitrogen attains its maximum. When hydrolysis is continued beyond this point, a transformation of amino nitrogen into ammonia follows, indicating a deaminization of some amino-acid or acids. The point at which the amino nitrogen reaches a maximum, with the least possible formation of ammonia, they define as the *end point* of the hydrolysis.

From the results of acid hydrolyses performed under various conditions Henriques and Gjaldbaek conclude that the most certain method for obtaining the above end point is treatment with 3*N* hydrochloric acid in an autoclave at 150° for one and one-half hours. With casein, Witte peptone and calves' flesh, complete hydrolysis could also be obtained by boiling with 20 per cent hydrochloric acid for twelve hours. With egg albumin, however, the results were different. The amount of amino nitrogen present after six hours' boiling with 20 per cent acid was only 89 per cent of that obtained by autoclave hydrolysis, and continuation of the boiling for six hours more resulted in no increase. From this it appeared that, in egg albumin at least, some peptid linkings exist in such an unusually stable condition that they can be split only by the autoclave hydrolysis.

Both Osborne and Guest² and the writer³ have based protein analyses on the assumption that hydrolysis is complete when the amino nitrogen set free by boiling with 20 per cent hydrochloric

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¹ *Zeitschr. f. physiol. Chem.*, lxvii, p. 8.

² This *Journal*, ix, p. 335; ix, p. 425.

³ *Ibid.*, x, p. 15, 1911.

acid reaches a maximum (sometimes attained only after twenty-four hours' or more boiling). The results of Henriques and Gjaldbaek with egg albumin necessitate a test of the validity of this assumption.

EXPERIMENTAL.

Portions of 1.5 grams of each protein, in air-dry condition, were placed in test tubes of 100 cc. capacity. To the tubes used in autoclave experiments 50 cc. portions of 3*N* hydrochloric acid were added; for experiments at 100° the same volume of 20 per cent. hydrochloric acid was used. The tubes for autoclave hydrolysis were warmed in a water bath for a short time when necessary in order to bring the proteins completely into solution before placing them in the autoclave. The latter was placed in an oil bath at 175°. When the bath and autoclave reached equilibrium the temperature had fallen to 150°, and it was maintained at this point for the one and one-half or three hours' duration of the experiment, at the end of which the autoclave was removed from the bath and allowed to cool in the air. The tubes for hydrolysis at 100° were loosely stoppered and were immersed in a bath of boiling water for intervals of ten, twenty-four, or forty-eight hours.

After hydrolysis the hydrochloric acid was distilled off as completely as possible under diminished pressure. The ammonia was then determined by distillation at 15–20 mm. pressure with calcium hydrate and alcohol, as described in the writer's method for analysis of proteins.⁴ The undissolved calcium hydrate was filtered off and the adsorbed melanin determined by the Kjeldahl method.⁵ The filtrate was neutralized with hydrochloric acid, concentrated under diminished pressure and brought to 100 cc. Duplicate portions of 20 cc. were used for Kjeldahl determinations, and 10 cc. portions for estimation of amino nitrogen by the nitrous acid method.⁶ The difference between the Kjeldahl and NH_2 determinations gives the non-amino (NH) nitrogen. This includes one NH_2 group, that of the guanidine nucleus of arginine, which does not react with nitrous acid. The results are given in percentages of the total nitrogen of the proteins.

TABLE I.

Casein.

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	150°	150°	160°
TIME OF HYDROLYSIS	10 hrs.	24 hrs.	48 hrs.	1½ hrs.	3 hrs.	3 hrs.
NH_2	8.9	9.4	10.1	9.8	9.8	12.1
NH_2^*	65.2	72.4	72.1	72.6	71.1	67.9
NH.....	23.8	16.1	16.1	16.3	17.0	18.0
Melanin.....	2.1	2.1	1.8	1.3	2.0	2.0

* Osborne and Guest (*This Journal*, ix, p. 334) found 71.7 per cent of nitrogen present as NH_2 in casein completely hydrolyzed by boiling 48 hours with 20 per cent HCl.

⁴ *This Journal*, x, p. 20.

⁵ *Ibid.*, x, p. 21.

⁶ *Ibid.*, ix, p. 185.

TABLE II.

Edestin.

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	110°*	150°	150°	160°
TIME OF HYDROLYSIS	10 hrs.	24 hrs.	48 hrs.		1½ hrs.	3 hrs.	3 hrs.
NH ₃	8.7	9.1	9.9	10.0	9.8	12.9	15.4
NH ₂	53.5	61.9	62.8	61.1	60.3	61.6	59.3
NH.....	36.2	27.5	25.3	26.3	27.0	24.5	24.3
Melanin....	1.8	1.4	2.1	1.9	2.9	1.0	1.0

* Results at 110° are taken from figures published in the paper on Analysis of Proteins, this *Journal*, x, p. 15. The proteins were boiled with 20 per cent hydrochloric acid twenty or more hours, until the amino nitrogen became constant.

TABLE III.

Wheat Gliadin.

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	110°†	150°	150°
TIME OF HYDROLYSIS	10 hrs.	24 hrs.	48 hrs.		1½ hrs.	3 hrs.
NH ₃	23.7	24.4	24.8	25.5	24.5	25.6
NH ₂ *.....	51.3	57.3	57.1	57.2	56.9	57.1
NH.....	22.7	16.1	16.0	16.3	16.4	17.2
Melanin.....	2.3	2.2	2.1	0.9	2.1	0.2

* Osborne and Guest (This *Journal*, ix, p. 425) obtained 59.2 per cent nitrogen in amino form in completely hydrolyzed gliadin. Our somewhat lower figure is probably due to a difference in the gliadin preparation used.

† Results at 110° are taken from figures published in the paper on Analysis of Proteins, this *Journal*, x, p. 15. The proteins were boiled with 20 per cent hydrochloric acid twenty or more hours until the amino nitrogen became constant.

TABLE IV.

Egg Albumin (Merck).

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	150°	150°
TIME OF HYDROLYSIS	10 hrs.	24 hrs.	48 hrs.	1½ hrs.	3 hrs.
NH ₃	5.5	6.0	7.7	7.5	12.0
NH ₂	69.4	79.5	78.0	78.3	73.4
NH.....	21.9	12.0	11.6	11.7	13.2
Melanin.....	3.2	2.6	2.7	2.5	1.4

TABLE V.
Ox Haemoglobin.

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	110°	150°	150°
TIME OF HYDROLYSIS	10 hrs.	24 hrs.	48 hrs.		1½ hrs.	3 hrs.
NH ₃	3.7	3.9	4.2	5.2	4.6	5.1
NH ₂	68.9	76.1	75.9	74.0	75.9	74.9
NH.....	22.0	14.9	15.3	16.6	14.3	15.3
Melanin.....	5.3	5.2	4.7	3.6	5.3	4.9

TABLE VI.
Wheat Gluten.

TEMPERATURE OF HYDROLYSIS	100°	100°	100°	150°	150°
TIME OF HYDROLYSIS	10 hrs.	24 hrs.	48 hrs.	1½ hrs.	3 hrs.
NH ₃	17.5	17.8	18.3	18.3	20.8
NH ₂	55.9	62.5	64.9	64.9	63.0
NH.....	23.5	16.7	14.3	14.1	14.3
Melanin.....	3.1	2.9	2.5	2.6	1.8

SUMMARY.

The percentage of amino nitrogen reaches a definite maximum when acid hydrolysis of a protein is complete, and this maximum is the same whether the hydrolysis occurs at 100° or at 150°. Approximately the same results are obtained by heating at 100° with 20 per cent hydrochloric acid for forty-eight hours as by heating in an autoclave with 3N acid for one and one-half hours as recommended by Henriques and Gjaldback.⁷ At 100° the amino nitrogen reached its maximum within ten hours in no case, but within twenty-four hours in every case, except that of gluten, which showed a small but definite increase (2.4 per cent) during the second twenty-four-hour period. It appears that with less acid than here used (35 parts of 20 per cent HCl to 1 part of protein) hydrolysis may be somewhat slower, as Osborne and Guest found twenty-four hours' boiling with 10 parts of 20 per cent HCl insufficient to completely hydrolyze casein.

The ammonia, as found by Henriques and Gjaldback, does not reach an absolutely definite maximum, but increases the longer hydrolysis is continued. At 150°, prolonging the hydrolysis beyond

⁷ *Loc. cit.*

one and one-half hours caused, in the cases of three of the six proteins, an increase of 2.5–4.5 per cent in the ammonia nitrogen. At 160° the increase of ammonia is still more marked and occurs at the expense of the amino nitrogen. There is much less tendency towards deaminization at 100°; the second twenty-four-hour period caused an increase of over 0.8 per cent in the ammonia only in the case of egg albumin (1.7 per cent). Henriques and Gjaldbaek have shown that the well known⁸ alteration which cystine undergoes when boiled with hydrochloric acid is accompanied by deaminization. That any of the other natural amino-acids are deaminized to an appreciable extent, unless heated under pressure, appears doubtful. That the ammonia, or “amide nitrogen,” arises chiefly from the acid amide groups of the asparagine and glutamine in the protein molecule, as indicated by the results of Osborne, Leavenworth and Brautlecht,⁹ is consistent with all present results.

⁸ Mörner: *Zeitschr. f. physiol. Chem.*, xxxiv, p. 207; Van Slyke: this *Journal*, x, p. 38.

⁹ *Amer. Journ. of Physiol.*, xxiii, p. 194, 1908.

GASOMETRIC DETERMINATION OF FREE AND CONJUGATED AMINO-ACIDS IN THE URINE.*

BY P. A. LEVENE AND DONALD D. VAN SLYKE.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

The methods for the gasometric determination of total (free + conjugated¹) and free² amino-acid nitrogen in the urine have been outlined in preliminary communications by one of us. The present paper describes the methods in detail, gives some comparative results with the gasometric and formol methods, and presents the results obtained with a number of normal and pathological human urines and with the urines of some dogs under unusual experimental conditions.

For the determination of the total (free and conjugated) amino-acid nitrogen of the urine the gasometric method has decided advantages over the well known Sørensen formol titration.³ Both methods require heating with acid to hydrolyze the conjugated amino-acids (hippuric acid, peptone, etc.). In the actual determination, however, the gasometric method has an inherent advantage, in that it offers a sharply defined volume of nitrogen gas to be measured at a single burette reading; while the formol titration necessitates the determination of two end points, one with litmus and the other with phenolphthalein, neither point being so sharply defined as those commonly used in volumetric work. The formol titration is further complicated when, as here, the solution to be analyzed is dark in color. This, of course, does not interfere with the gasometric determination. The latter possesses another advantage in accuracy, particularly when small amounts are to be determined, in that the volume of nitrogen gas evolved is five times

* Received for publication, July 2, 1912.

¹ Van Slyke: *Ber. d. deutsch. chem. Gesellsch.*, xliii, p. 3179.

² *Ibid.*, xliv, p. 1685.

³ *Biochem. Zeitschr.*, vii, p. 44.

that of the N/5 barium hydroxide solution required to titrate the same amount of amino nitrogen. To the formol method also attaches the theoretical objection, pointed out by its originators, that the results will be raised by the presence of weak organic acids, such as oxybutyric, which require more alkali to neutralize them to phenolphthalein than to litmus paper. Whether the error from this source is large enough to affect the significance of results is still undetermined.

For the determination of free (unconjugated) amino-acids, on the other hand, the formol method has a great advantage over the gasometric in that the former is not affected by the presence of urea. Urea, which we have been unable to remove without either hydrolyzing conjugated amino-acids or removing free ones, reacts slowly with nitrous acid, so that a correction has to be introduced for it into the gasometric results. Although this correction can be determined with a fair degree of accuracy, the proportion of urea to amino-acid nitrogen is so great (50 or 100 to 1) that the necessity for the correction more than offsets the advantages which the gasometric method possesses in the absence of urea or the presence of relatively small amounts. A possible error of ± 0.5 per cent of the total nitrogen of the urine must be allowed for. As the free amino-acid nitrogen normally constitutes only about 1 per cent of the total nitrogen of the human urine, it is evident that the gasometric method is here of value only for determining an abnormally high content of amino-acids. For the detection of a significant increase it is adequate. It also possesses the advantage, over the formol method, that it cannot be affected by the occurrence of abnormally large amounts of organic acids in the urine. For this reason we have described a form of the gasometric method which we have found practicable, although it is not ordinarily so accurate as the formol method for free amino-acid nitrogen, especially in the form in which the latter has been recently developed by Benedict and Murlin.⁴

⁴ *Proceedings of the Soc. of Exp. Biol. and Med.*, ix, May, 1912.

DETERMINATION OF TOTAL (FREE AND CONJUGATED) AMINO-ACID NITROGEN IN THE URINE.

In this estimation hippuric acid, peptides, albumin, etc., are hydrolyzed, while urea is decomposed into ammonia and carbon dioxide by heating with dilute sulphuric acid under pressure. The ammonia is then boiled off, removing both the preformed ammonia and that from the decomposed urea, and the amino nitrogen is determined in the residual solution. One can obtain very accurate results with this method.

To 75 cc. of urine in a large test tube one adds 2.5 cc. of concentrated sulphuric acid. The urine is then heated in an autoclave at 175° for an hour and a half.⁵ It is then transferred to a 300 cc. Jena Erlenmeyer, 5 to 6 grams of powdered calcium hydrate are added, with a piece of paraffin to prevent foaming, and the mixture is boiled until the vapors no longer turn red litmus blue. If necessary, water is added to keep the solution from going dry before this point is reached. Usually all the ammonia is driven off within about an hour, but sometimes a urine is encountered which requires twice as long.

The hydrolyzed urine is filtered through a folded filter into an evaporating dish, the precipitate, consisting of calcium sulphate, hydrate, etc., being washed ten times with hot water. The filtrate is concentrated on the steam bath to a volume of a few cc. and filtered from the small amount of calcium salt, which has settled out into a 25 cc. measuring flask. One can then perform duplicate amino determinations in the usual way⁶ on 10 cc. portions. The total amino nitrogen in normal urines appears to be quite constant at 1.5 to 2.5 per cent of the total nitrogen. More than 3 per cent we have encountered only in pathological cases, in which the high results are usually due to albumin.

About a full working day is required by the above method to prepare the urines for the final determination; but during a large part

⁵ The quantitative decomposition of urea under these conditions was demonstrated by Benedict and Gephart (*Journ. Amer. Chem. Soc.*, 1909) and confirmed by Levene and Meyer (*ibid.*). The new method of Benedict (decomposition of the urea in a KHSO_4 melt) is not applicable here.

⁶ Van Slyke: this *Journal*, ix, p. 185; cf., also, preceding article on improvements in the method.

of the time (heating in autoclave, concentrating on water bath) but little attention is required. As many urines can be prepared at the same time as the autoclave will hold tubes, the ammonia from all being boiled off at the same time on a hot plate.

The following results indicate the accuracy of the method. Two 75 cc. portions of normal urine were analyzed as described, 0.1835 gram of Kahlbaum's alanine having been added to one portion, the other serving as control. The solutions, at the end of the analysis, were brought to 25 cc. and the amino nitrogen determined, as usual, in 10 cc. portions.

	N	TEMPER- ATURE	PRESSURE	NH ₄ -N	ALANINE N FOUND	ALANINE N PRESENT
	cc.	degrees C.	mm.	mgm.	mgm.	mgm.
Urine I.	9.0	21	758	5.09		
Urine II.	9.0	21	758	5.09		
Urine—Alanine I.	29.4	20	757	16.67	11.58	11.55
Urine—Alanine II.	29.1	19	756	16.56	11.47	11.55

The fact that the autoclave treatment effects a practically complete cleavage of hippuric acid is shown by the following. One gram of sodium hippurate was dissolved in 75 cc. of water with 2.5 cc. of concentrated sulphuric acid and heated one and a half hours in the autoclave. The solution was then diluted to 100 cc. and determinations of the total nitrogen present made upon 20 cc. portions, those of the amino nitrogen upon 10 cc. portions.

TOTAL NITROGEN		AMINO NITROGEN	
Found	Calculated	Found	Calculated
0.0693	0.0696	0.0685	0.0696

The complete hydrolysis of a soluble protein effected by the autoclave treatment is shown by the following results. One gram of air-dried proto-albumose was dissolved in 75 cc. of water, the remainder of the treatment being that described above in the hippuric acid experiment. The total nitrogen found was 0.1484 gram; the amino nitrogen was 0.1025 gram, or 69.1 per cent of the total. In proto-albumose, completely hydrolyzed with boiling hydrochloric acid, 69.13 per cent of nitrogen in amino form was found.⁷ While

⁷ Levene, Van Slyke and Birchard: this *Journal*, x, p. 68, 1911.

all proteins may not be hydrolyzed with the same degree of absolute completeness, it may be stated with confidence that the amount of unhydrolyzed albumin left in a urine after the autoclave treatment for destruction of urea will be minimal.

DETERMINATION OF FREE AMINO-ACID NITROGEN.

The amino-acids, when treated with nitrous acid under the conditions used for amino nitrogen determination,⁸ give off in two to five minutes 100 per cent of their nitrogen. Urea under the same conditions requires about eight hours to react completely. In two to five minutes it gives off only 3-4 per cent of its nitrogen, and the reaction continues for a considerable time without appreciable change in the volume of nitrogen evolved per minute. This rate is also unaffected by the presence of reacting amino-acids. By taking advantage of the slow and regular rate at which urea reacts, one can determine in its presence the nitrogen of the almost instantaneously reacting amino-acids, making a correction for the small percentage of urea which reacts within the short time required for the determination.

The ammonia must first be removed, and the determinations of NH_3 - and NH_2 -nitrogen can be conveniently combined. We found distillation with calcium hydrate under diminished pressure the most satisfactory means for removing and determining the ammonia. The apparatus and technique used are the same as described for the determination of amid nitrogen in proteins.⁹ Fifty or 100 cc. of urine, with an equal volume of 95 per cent alcohol and enough of a 10 per cent calcium hydrate suspension to give a strongly alkaline reaction, are placed in a double-necked distilling flask of 500 cc. or 1 liter capacity, and distilled as described, until all the alcohol has been driven off. This point is reached in twenty to thirty minutes and is indicated by vigorous foaming of the solution. It is essential that the removal of the ethyl alcohol be practically complete; as when mixed with nitrous acid it gives off vapors or gases which are only with difficulty absorbed by the permanganate solution.

⁸ Van Slyke: Quantitative Determination of Aliphatic Amino Groups, this *Journal*, ix, p. 185; xii, p. 275.

⁹ Van Slyke: this *Journal*, x, p. 21.

The distillation being finished, the calcium hydrate and salts are dissolved by the addition of 2–3 cc. of glacial acetic acid. The urine is now transferred to a measuring flask and made up to its original volume of 50 or 100 cc. Of this, 10 cc. portions are used for the amino determination. It is of no advantage, except in unusually dilute urines, to concentrate the urine so that relatively more can be used for the determination. The limit of accuracy of the determination depends upon that of the urea correction, and the absolute error of this is increased in proportion to the concentration of the urea, so that there is little or nothing to gain in percentage accuracy by increasing the volume of gas to be measured.

The amino determination and the correction for the urea are now made as follows. One amino determination is performed, the duration of the reaction being the shortest time in which, at the prevalent temperature, amino-acids will react completely. Immediately thereafter the determination is repeated under the same precise conditions, except that the reaction is continued for exactly twice as long. The *increase* in volume obtained, by doubling the reaction time, represents the amount of nitrogen evolved from the *urea* during half the time of the second determination, or, the entire duration of the first. It is, therefore, the correction which must be subtracted from the first result in order to leave only the nitrogen obtained from amino-acids. Amino-purines and amino-pyrimidines react slowly, like urea, and consequently if present will not be determined with the amino-acids.

In the preliminary description of the method, when the original hand-manipulated apparatus was employed, six and twelve minutes were the durations used for the two determinations. The more efficient action of a rapidly shaken motor-driven apparatus enables one to cut the time down to the following: for temperatures of 15 to 20°, four and eight minutes; for 20 to 25°, three and six minutes; for over 25°, two and four minutes. During the shorter of the two determinations the reacting solution is shaken constantly and rapidly. During the longer determination it may either be shaken constantly, or, during the first half minute (to mix the solutions) and the last two minutes (to drive out all the nitrogen formed). It is particularly essential that the shaking should be

rapid during the last minute, in each case, so that the nitrogen formed up to the end of the interval may be at once driven out.

The volumes of acetic acid and nitrite solution used and the volume of nitrous acid solution in the deaminizing vessel when the urine is run in must here be accurately the same in each pair of determinations, and not roughly measured, as may be the case in ordinary amino analyses. Exact measurement of the reaction time is also essential and is easy to attain with a stop watch. In order to make the starting points for the time measurement as sharp as possible, we run the urine into the deaminizing vessel rapidly until only a few tenths of a cubic centimeter remain to be added. The watch is then started, while the remaining small portion of the solution is added more cautiously.

The approximate constancy of the reaction rate of urea during at least the first eight minutes, at a given temperature, is illustrated by the following figures. The solution used contained 2 per cent of urea, about as much as an average human urine.

DURATION OF REACTION	TEMPERATURE	N	N PER MINUTE
<i>minutes</i>	<i>degrees C.</i>	<i>cc.</i>	<i>cc.</i>
3	25	9.8	3.27
6	25	19.5	3.25
4	25	13.2	3.30
8	25	26.2	3.28
3	19	5.8	1.93
3	19	6.0	2.00
6	19	12.0	2.00
6	19	11.6	1.93

The following determinations indicate that approximate results for amino-acid nitrogen can be obtained in the presence of a great

DURATION OF REACTION	TEMPERATURE	PRESSURE	N	CORRECTION FOR UREA N	N FROM ALANINE	CALCULATED N FROM ALANINE
<i>minutes</i>	<i>degrees C.</i>	<i>mm.</i>	<i>cc.</i>		<i>cc.</i>	<i>cc.</i>
3	24	752	22.6	8.7	13.9	14.2
6	24	752	31.3			
3	24	752	23.1	8.5	14.6	14.2
6	24	752	31.6			
3	24	752	23.3	8.4	14.9	14.2
6	24	752	31.7			
2	29	760	23.8	9.7	14.1	14.3
4	29	760	33.5			
2	29	760	23.6	9.7	13.6	14.3
4	29	760	33.3			
2	29	760	23.8	9.6	14.2	14.3
4	29	760	33.4			

excess of urea, making the correction for urea in the manner described above. The solution used contained 2 grams of urea and 0.5 gram of alanine per 100 cc. The proportion of urea nitrogen to amino-acid nitrogen was 12:1. Three pairs of determinations were performed on each of two different days, on which there were 5° difference in the room temperatures. The solution was kept at 0° during the interval in order to prevent the rapid deamination which bacteria can accomplish.

As a further control, 200 cc. of normal human urine, containing 0.574 gram of nitrogen per 100 cc., were freed from ammonia as previously described (ammonia nitrogen = 0.021 gram per 100 cc.). Determination of the free amino nitrogen in this urine showed that the amount present was too small to detect. Two-minute determinations at 29° gave 4.0, 4.0, 4.3 cc. of N; four-minute determinations gave 8.1, 8.4, 8.4 cc. One half gram of alanine was dissolved in 100 cc. of the urine, which was then freed from ammonia, brought back to 100 cc. and used for the following determinations. The temperature was 19°, pressure, 760 mm.

DURATION OF REACTION	N	N FROM AMINO-ACIDS	CALCULATED FOR ALANINE PRESENT	ERROR
<i>minutes</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
3	17.8	13.5	13.8	-0.3
6	22.1			
3	18.0	13.3	13.8	-0.5
6	22.7			
3	18.3	14.1	13.8	+0.3
6	22.5			

ANALYSES OF HUMAN NORMAL AND PATHOLOGICAL URINES.

The analyses tabulated were carried out as described above except that most of the determinations of free amino-acid nitrogen were performed by the earlier modification¹⁰ used before the improved apparatus for determining amino nitrogen¹¹ was available. When the results for total amino-acid nitrogen were checked with Sørensen's formol method, 10 cc. of the 25 cc. of final solution were used for the gasometric determination and an equal portion for the formol titration. The formol results, though in some cases not appreciably different from the gasometric, were as a rule mark-

¹⁰ *Ber. d. deutsch. chem. Gesellsch.*, xlv, p. 1685, 1911.

¹¹ *This Journal*, xii, p. 275.

edly higher, the maximum difference being equal to 1 per cent of the total nitrogen of the urine.

The higher results for the formol method may be due to the presence of organic acids, which are prejudicial to its accuracy. It appears doubtful that, as a rule, this source of error is sufficient to cause a clinically or scientifically significant deviation in the formol results, although a positive statement can not be made until the formol and gasometric methods have been used together on a larger and more varied collection of urines.

The total (free + conjugated) amino-acid nitrogen bears a fairly constant relation to the total nitrogen. All the normal and most of the pathological urines contain not less than 1.1 nor more than 2.8 per cent of their nitrogen in this form. The only cases in which 3 per cent was exceeded were those of nephritis and the arthritic cases, which also usually have albumen in the urine. The probability that all the abnormal amino nitrogen obtained was freed by cleavage in autoclave is shown by the later cases, in which the free amino nitrogen was also determined. This is not high even when the total amino nitrogen of a nephritic reaches 24 per cent. Aside from nephritic cases, one cancer case gave a very high figure, 6.07 per cent. Unfortunately we had no determination of the free amino nitrogen in this case. Two cases of liver cirrhosis gave entirely normal values.

The free amino nitrogen never exceeded 1.5 per cent in the limited number of cases in which it was determined.

NO.	CASE	TOTAL N PER 100 CC. URINE	TOTAL NH ₂ PER 100 GRAMS N (Gasometric)	TOTAL NH ₂ PER 100 GRAMS N (Formol)	FREE NH ₂ PER 100 GRAMS N (Gasometric)
1	Normal.....	0.300	2.50		
2	Normal.....	0.808	1.95	2.45	
3	Normal.....	0.925	1.80	2.76	
4	Normal.....	0.780	1.86	2.45	
5	Normal.....	1.336	2.02	2.42	
31	Normal.....	0.941	1.77		0.3
35	Normal.....	1.043	1.14		
36	Normal.....	0.623	2.81		1.5
37	Normal.....	0.491	2.12		0.3
38	Normal.....	1.470	1.28		0.5
39	Normal.....	0.613	2.70		0.5
40	Normal.....	0.624	2.41		0.2
50	Normal.....	1.067	1.28		
5	Cancer of antrum.....	0.347	2.10		
32	Carcinoma of breast.....	0.702	6.07		

NO.	CASE	TOTAL N PER 100 CC. URINE	TOTAL NH ₂ PER 100 GRAMS N (Gasometric).	TOTAL NH ₂ PER 100 GRAMS N (Formol).	FREE NH ₂ PER 100 GRAMS N (Gasometric).
44	Carcinoma of larynx	1.485	2.29		1.5
51	Carcinoma of larynx	1.382	2.38		
2	Arthritis	0.585	2.39		
6	Arthritis	0.580	5.56		
11	Arthritis	0.363	3.25		
12	Arthritis	1.201	3.60	3.97	
13	Arthritis	1.112	2.26	2.34	
14	Arthritis	0.901	2.17	2.73	
15	Arthritis	0.454	1.90		
20	Gout	0.568	3.87	3.89	
1	Multiple sclerosis	0.655	1.65		
7	Multiple sclerosis	0.396	2.51		
8	Multiple sclerosis	0.943	1.32		
3	Paralysis agitans	1.813	1.54		
16	Anterior poliomyelitis	1.283	1.34	1.83	
46	Syphilis	0.745	2.92		
55	Hypophysis disease	0.364	1.55		
4	Progressive musc. dystrophy	0.728	1.92		
9	Progressive musc. dystrophy	1.654	1.82		
17	Progressive musc. dystrophy	0.959	2.33	2.48	
33	Progressive musc. dystrophy	1.470	1.83		
41	Progressive musc. dystrophy	1.267	2.03		1.1
47	Progressive musc. dystrophy	0.415	1.82		1.5
49	Progressive musc. dystrophy	1.923	1.37		0.5
52	Progressive musc. dystrophy	1.210	1.61		0.3
54	Progressive musc. dystrophy	0.434	1.21		0.8
34	Liver cirrhosis	0.889	1.92		0.6
45	Liver cirrhosis	0.455	1.68		0.8
42	Nephritis	0.781	2.00		1.2
43	Nephritis	0.654	24.30		1.2
53	Nephritis	1.497	7.53		2.0
56	Nephritis	0.364	1.55		
57	Nephritis	1.120	1.62		

Analyses of Dog Urines.

	TOTAL N PER 100 CC. URINE	TOTAL NH ₂ PER 100 GRAMS N (Gasometric)	TOTAL NH ₂ PER 100 GRAMS N (Formol)	FREE NH ₂ PER 100 GRAMS N (Gasometric)
Normal dog (urine diluted)	0.625	1.67	1.79	
Normal dog (urine diluted)	1.294	1.05	0.92	
Dog fed glycine anhydride (urine diluted) ..	0.168	39.80	39.20	
*Dog poisoned 10 days with chloroform	2.711	1.78		
*Dog poisoned 11 days with chloroform	1.523	1.92		
*Dog poisoned with chloroform and phosphorus (urine diluted)	0.893	1.59		
Normal dog fed 2 grams <i>dl</i> -phenylalanine (urine diluted)	0.226	8.50		
Normal dog fed <i>dl</i> -aspartic acid (urine diluted)	0.125	2.38		
Gastrectomized dog, normal diet (urine diluted)	0.188	1.91		

	TOTAL N PER 100 CC. URINE	TOTAL NH ₃ PER 100 GRAMS N (Gasometric)	TOTAL NH ₃ PER 100 GRAMS N (Formol)	FREE NH ₃ PER 100 GRAMS N (Gasometric)
Gastrectomized dog, normal diet (urine diluted).....	0.406	1.90		
Gastrectomized dog, normal diet (urine diluted).....	0.219	2.78		
Gastrectomized dog, normal diet (urine diluted).....	0.415	2.02		
Dog which had received 12 grams of <i>dl</i> -alanine intravenously 20 minutes before catheterizing.....	0.445	53.00		52.40

* Urine furnished by Dr. Dochez.

It was a cause for surprise that the dogs poisoned with chloroform, and with chloroform and phosphorus, gave urines containing only the normal small percentage of amino nitrogen, although the livers of these animals were degenerated to an extreme degree. These results indicate that even rapid degeneration of the liver may not result in excretion of amino-acids. The two cases of human liver cirrhosis also showed only normal amino nitrogen in the urine.

The non-utilizability of glycine-anhydride in the organism is strikingly shown by the tremendous excretion of conjugated amino-acid nitrogen following its administration. The permeability of the kidney for amino-acids, when injected directly into the blood, is shown by the last result. It does not indicate that the organism fails to utilize amino-acids even thus injected, however, for only 1.5 grams of the 12 grams of alanine injected were excreted unchanged.

NITROGEN AND NUCLEIN METABOLISM IN GOUT.*

By P. A. LEVENE, M.D., AND LEO KRISTELLER, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research and
from the Montefiore Hospital, New York.)*

For more than a century the pathology of gout has been associated with disturbances of uric acid metabolism. When the first theories of gout were advanced, there existed very little exact knowledge of the chemistry of uric acid and still less of its physiology. Every step in the progress of our knowledge of the chemistry of purin bodies, and of the biology of nuclein derivatives, led to a revision of the older theories of gout. A critical review of the most important views on this pathological condition has been published by Brugsch and Schittenhelm¹ and need not be repeated in this place.

The work of these authors was done in the light of the latest achievements in the biochemistry of nuclein compounds, and as a result chiefly of their own experiments they proposed a new theory to explain the disease. The principal advance made by these investigators is due to the fact that they placed their patients on a purin-free diet, and owing to this they were able to differentiate the process of endogenous from that of exogenous nuclein metabolism. They arrived at a conclusion similar to that of older writers; namely, that the pathology of gout is closely associated with that of nuclein metabolism. According to their view the disease is characterized by three conditions: first, retarded uric acid formation; second, retarded uric acid disintegration; and finally, retarded uric acid elimination.

The considerations which led them to formulate their theory were the following: the uric acid output of a gouty individual on a purin-free diet was lower than the output of a normal man under the same conditions. On the other hand, in the course of the disease the average uric acid content of the blood was higher than in health. If, however, purin bases, or nuclein material, were added to the purin-free diet, the increase in the elimination of uric acid of the gouty individual was lower in quantity, and at the same time more protracted than in health. A normal individual, on a purin-free diet after the intake of nuclein material, responded with a prompt increase in the output of uric acid, but this increased output had a very short duration.

* Received for publication, May 24, 1912.

¹ Brugsch, T. B., and Schittenhelm, A. S., *Der Nukleinstoffwechsel und seine Störungen*, Jena, 1910.

This difference in the reaction of the organism to ingested purin suggested to Brugsch and Schittenhelm the view that the process of uric acid formation in the gouty organism was retarded as compared with that in normal individuals. Nevertheless, the absolute quantity of uric acid destroyed by the patient was higher than that destroyed by the normal individual, for the reason that at any given time the tissues of the patient contained more uric acid, and although its destruction at a given place was progressing with a lower intensity, more uric acid was destroyed in the body as a whole in a given period of time. The fact that the tissues and the blood of gouty individuals do not contain more uric acid than is generally found in them is the result of this arrangement.

Although the views of Brugsch and Schittenhelm met with some opposition, still in the main they were accepted. However, these authors neglected to include in the sphere of their observation the course of elimination of nitrogenous substances of non-nuclein origin. Hence it is possible that the abnormalities which they found in connection with nuclein metabolism may exist as a general abnormality of metabolism of all nitrogenous substances. The present investigation was undertaken with a view of obtaining light on this point and also to extend the knowledge of the progress of nucleic acid disintegration in the course of gout.

In a previous study on chronic granular nephritis the authors² observed that in that disease all nitrogenous end-products of metabolism were retarded in their elimination when the daily intake contained more nitrogen than the diseased kidneys could conveniently eliminate. However, if the eliminating capacity of the kidneys was taken into consideration, and the nitrogen intake regulated on the basis of this capacity, then the character of elimination in nephritis had a course similar to that in health.

For this reason it seemed to us of importance to begin the study of metabolism in the following manner: first, to establish the normal eliminating capacity of the kidneys for nitrogenous end-products of metabolism; second, to place the patient on a diet not exceeding this eliminating capacity; third, to compare the normal progress of nitrogenous output with that obtained after administration of additional nitrogenous substances derived from nucleic acid, or from proteins.

²Levene, P. A., Kristeller, L., and Manson, D., *Jour. Exper. Med.*, 1909, xi, 825.

The observations recorded here were made on a patient in a very advanced stage of gout. Very large gouty deposits in nearly all joints prevented him from any active movements. The patient was at first placed on a daily diet containing about six grams of nitrogen. When the daily output became fairly constant (about five and one half grams), the experiments were begun. These consisted in the addition of urea to the normal diet. The urea was added to the first morning meal and the nitrogen output was followed in twenty-four hour periods, day by day. In a similar manner experiments were carried out with asparagin, plasmon, uric acid, and nucleic acid.

It was observed that after the administration of very simple nitrogenous substances, such as urea and asparagin, the patient did not remove the surplus nitrogen in the same manner as a normal individual, or even as the patient with granular nephritis who was placed on a low nitrogen intake. The increase in the nitrogen output was comparatively low during the first twenty-four hour period, and this slightly increased excretion usually continued for several days.

This suggested a comparison of the character of nitrogen elimination when the patient was placed on a daily diet containing about thirteen grams of nitrogen. On this diet, after administration of urea, the rise in the nitrogen output took place with greater rapidity and with greater intensity than on the low protein diet. Also the nitrogen output in the intervals between the experiments appeared to be more uniform than when the patient had been on a low protein intake.

For this reason it was decided to perform a new series of experiments, placing the patient on a diet abundant in nitrogen. But even under these conditions the increase in the output of nitrogen, after the administration of additional nitrogenous substances, was very low and protracted. This made it difficult to detect the differences in the output during the intervals between experiments and during the days of the experiments.

A new series of experiments was then performed, in the course of which the additional nitrogenous products were added, not during one day, but during three or four consecutive days, and the output of nitrogen was followed in twenty-four hour periods for a

number of days. But this change in daily nitrogen intake brought little change in the results of the experiments, for the increase in nitrogen output after the administration of nitrogenous substances was rather insignificant, and the excretion was very often protracted. For the sake of convenience, therefore, it was decided to estimate the increase in the nitrogen output during the days of the experiment and during the two days following the experiment.

While the patient was under observation, which was for three winter terms, Medigreceanu and one of us³ noticed that after the administration of purin bodies, the nitrogen output in animals had a more regular course if they received simultaneously considerable quantities of sodium bicarbonate. It was, therefore, decided to repeat the experiment on our patient, administering to him daily fifteen grams of this salt. It seemed that under these conditions the patient removed the surplus nitrogen intake with greater rapidity than without the bicarbonate of soda.

Together with the study of the character of the general nucleic metabolism an attempt was made to obtain information regarding the manner in which nucleic acid underwent disintegration in the organism of the gouty individual, with a view to ascertaining whether or not there existed any distinction in this regard between the gouty and the healthy organism.

It has been established in recent years that nucleic acids are composed of simple nucleotides, which in their turn consist of phosphoric acid and nucleosides. It has also been established recently that in the normal organism nucleic acid undergoes gradual decomposition, first into nucleotides, then into phosphoric acid and nucleosides, and finally into a base and a carbohydrate. Hence, it was planned to ascertain whether the complexity of the substance in which the purin bodies were administered to the organism had an influence on the rate and duration of the excessive nitrogen output. The patient received uric acid, hypoxanthine, inosine, guanosine, and nucleic acid in different experiments. For convenience of the analysis of the results of the experiments, the figures of the surplus output of nitrogen during the first twenty-four hours are presented in table I.

³ Levene, P. A., and Medigreceanu, F., *Am. Jour. Physiol.*, 1911, xxvii, 438.

TABLE I.

Experiments.	N of standard diet in gm.	N of additional substance in gm.	Per cent. of N of additional substance eliminated in the first twenty-four hours.
First series.....	7.0	2.5 (urea)	65.0
	7.0	2.5 (asparagin)	59.0
	7.0	2.5 (uric acid)	26.0
	7.0	2.5 (uric acid)	29.0
	7.0	1.85 (nucleic acid)	50.0
Second series.....	6.0	2.0 (urea)	0
	10.0	2.0 (urea)	57.0
	13.0	2.0 (urea)	70.0
	13.0	2.0 (nucleic acid)	34.0
	13.0	2.0 (uric acid)	0
	13.0	2.0 (uric acid)	29.0
Third series.....	6.0	2.0 (urea)	0
	6.0	2.0 (urea)	17.5
	13.0	2.0 (urea)	69.0
	13.0	2.0 (hypoxanthine)	50.0
	13.0	2.0 (hypoxanthine)	15.5
	13.0	1.1 (inosine)	60.0
	13.0	1.5 (guanosine)	23.0
	13.0	2.0 (uric acid)	40.0

The figures presented in the table do not show a great regularity in the removal of the surplus nitrogen ingested. It is clearly seen, however, that the removal of nitrogen ingested in the form of nucleic acid derivatives is lagging behind the nitrogen output after the administration of urea. The nearest to urea in its behavior is inosine, then follow nucleic acid, hypoxanthine, and the most imperfect elimination follows the administration of uric acid and of guanosine.

The rate of elimination of the surplus nitrogen is controlled by two factors, the rate of absorption and that of oxidation, and it is possible that the low figures observed after uric acid and guanosine intake are brought about by the comparative insolubility of these substances. Both inosine and hypoxanthine possess a comparatively high degree of solubility, and it appears as if the elimination of inosine nitrogen proceeds with greater rapidity.

It is not improbable that the factors of purin metabolism discovered by Walter Jones come into play in this instance. Jones⁴ discovered that oxidation of the nucleosides and of purin bases is brought about by different enzymes, and it is possible that in this instance the nucleoside-oxidases were more active than the purin-

⁴ Jones, W., *Jour. Biol. Chem.*, 1911, ix, 129, 169.

oxidases. This may also explain the reason for the more rapid nitrogen elimination after administration of nucleic acids. As a general rule, however, we noticed in our patient a comparatively slow rate of oxidation of nucleic acid derivatives, particularly of uric acid.

However, a review of the figures recording the surplus nitrogen output for the entire period of the experiments indicates that in the course of some days the greatest part of the nuclein nitrogen is removed from the body, although again in this respect there is noted a considerable irregularity.

The principal form in which the nitrogenous products of nucleic acid finally reappear in the urine seems to be urea. The increase in uric acid output and in the output of purin bodies is rather small, and the highest increase was noted after the administration of nucleic acid.

CONCLUSIONS.

Thus, our conclusions harmonize with those of Brugsch and Schittenhelm in so far as they are concerned with the nuclein metabolism. They add, however, the observation that the elimination of nitrogenous substances of protein origin has a protracted character, and that the oxidation of as simple a substance as asparagin proceeds at a subnormal rate.

The most striking peculiarity observed in our patient was the very imperfect elimination of ingested urea when the patient was placed on a diet containing only six grams of nitrogen per day, and a much more complete elimination when the diet contained thirteen grams of nitrogen.

EXPERIMENTAL PART.

Methods of Analysis.—Total nitrogen was estimated by the Kjeldahl-Gunning method; urea, originally by Folin's method, and later by the Levene and Meyer¹ modification of Benedict's process; ammonia, by the method of Shafer-Folin; uric acid, by the method of Ludwig-Salkowsky. Purin bases were estimated by treating the mother liquor from uric acid with mercuric sulphate, decomposing the precipitate, and reprecipitating the purin bases with ammoniacal silver solution.

Food Analysis.—All cereals were obtained in large quantities and a sample of each new supply was analyzed. Milk, cream, butter, eggs, and bread were analyzed from time to time. Vegetables were not analyzed, their composition being calculated on the basis of figures given by Koenig.

¹ Levene, P. A., and Meyer, G. M., *Jour. Am. Chem. Soc.*, 1909, **xxx**i, 717.

Diet.—The diet all through the experiments was purin-free. The patient was in charge of a special nurse who was familiar with the diet problem, and all meals were prepared by her personally. The diet consisted of eggs, milk, cream, butter, bread, cereals, vegetables, and fruit.

History.—The patient was Allen S., 44 years of age, a native of England. He had suffered from gout for twenty-seven years, and in the early period of the disease had frequent acute attacks. All his joints were deformed and stiff, and had been so since he had been under our observation.

The metabolism studies were carried out during the winter months of three consecutive years. With the beginning of warm weather the daily variations in nitrogen output were so great that the investigations had to be interrupted. Also in the course of the winter it generally required many weeks of a given diet before the nitrogen elimination approached a condition of equilibrium.

FIRST SERIES OF EXPERIMENTS.

During this series of experiments the additional substance was administered in one day periods and the nitrogen output was followed for several days after the administration. The standard output was calculated on the basis of an observation lasting eight days, from April 9 to April 16. The average output of total nitrogen was 6.72 gm. per day; of urea nitrogen, 5.68 gm., or 84.5 per cent. of the total; and of uric acid nitrogen, 0.081 gm., or 1.2 per cent. of the total.

The eliminating capacity of the kidney was determined by administration of urea. Two and one half gm. of nitrogen in the form of urea were given on April 26, 1909. During that day there were eliminated in excess over the standard days 1.63 gm. of nitrogen, and in the course of the three days that followed the administration, there were eliminated 3.67 gm. of nitrogen. The increase in urea nitrogen output for the same interval was 3.13 gm. and the average uric acid output per day was 0.078 gm. In previous observations on men and normal animals it was demonstrated that all of the urea administered is eliminated within the first twenty-four hours. In the present experiment during the same period only 65 per cent. of the excessive nitrogen intake was removed. Moreover, part of the excessive elimination was due to the diuretic effect of the substance. By this is explained the fact that of the excessive nitrogen output only 85 per cent. was in the form of urea. The daily output of uric acid for the same period did not differ materially from the normal.

Substances other than urea tested during this series were asparagin, uric acid, and nucleic acid.

Asparagin was given April 17, 1909. The additional nitrogen intake was 2.5 gm. The excessive output for forty-eight hours was 2.23 gm., the excessive output of urea nitrogen for the same period was 2.58 gm., and the daily output of uric acid nitrogen was 0.014 gm., which is not very significant. In previous observations on men and on normal animals it was found that the nitrogen of asparagin was removed with the same rapidity as urea; namely, all in the course of the first twenty-four hours. In this instance only 59 per cent. of the excessive intake was removed during that period.

Uric Acid was administered in two experiments. In the first experiment 2.5 gm. of nitrogen were given on May 6, 1909. During the following four days

there were eliminated 3.12 gm. of total nitrogen, 2.97 gm. of urea nitrogen, and 0.050 gm. of uric acid nitrogen in excess over the normal. The significant feature of this experiment is the fact that in the first twenty-four hours only 0.26 gm. of excessive nitrogen was removed, or 10 per cent. of the additional intake.

The second uric acid experiment was performed on May 10, 1909. The same quantity of uric acid was given. The excessive nitrogen output in the four days that followed was 3.23 gm., the excessive urea nitrogen was 3.63 gm., and the uric acid output 0.078 gm. per day, practically the same as on standard days. Again the significant feature of this experiment was the low nitrogen output in the first twenty-four hours; namely, only 29 per cent. of the excessive intake.

Nucleic Acid was administered on May 19, 1909. There were given 1.83 gm. of nitrogen in the form of nucleic acid. Following the second uric acid experiment the daily nitrogen output never came down to that of the original standard period. Therefore, the elimination after nucleic acid intake was compared, not with the first standard period, but with the average output of the five days preceding the nucleic acid intake. The daily average output for those five days was: total nitrogen 7.55 gm., urea nitrogen 6.61 gm., or 87.5 per cent. of the total, and uric acid nitrogen 0.0758 gm., or 1 per cent. of the total.

During the four days following the administration of nucleic acid there was an excessive output of 2.98 gm. of total nitrogen, 2.50 gm. of urea, and the uric acid nitrogen showed an increase of 0.0783 gm. It is noteworthy that in the first twenty-four hours the increase in the nitrogen output was over 50 per cent. of the excessive intake. Of course in part the increased nitrogen output during this period was occasioned by the diuresis produced by the absorption of nucleic acid.

SECOND SERIES OF EXPERIMENTS.

This series of observations lasted from November, 1909, to March, 1910. Again on this occasion an attempt was made to ascertain the most favorable conditions for the elimination of the nitrogen of the substances added in excess of the standard diet. The patient was placed in a condition approaching nitrogenous equilibrium on a diet containing less than 6 gm. of nitrogen. To this diet on the day of the experiment 2 gm. of nitrogen in the form of urea were added, but the nitrogen elimination remained unaffected by it. The nitrogen intake was then raised to 10 gm., and after the patient was placed in a condition of nitrogenous equilibrium, 2 gm. of nitrogen in the form of urea were added. But even then the increase in nitrogen elimination was very slow, and in one experiment the total increase in nitrogen output after an additional administration of 2 gm. of nitrogen was not much above 50 per cent. over the normal. The nitrogen intake was raised to 13 gm. per day and the urea administration proved satisfactory. Experiments were then performed with uric acid and with nucleic acid.

Experiments with an Intake of Six Grams of Nitrogen.—The normal output previous to the administration of urea was 5.98 gm. After the administration of an additional 2 gm. of nitrogen in the form of urea, on November 10, the nitrogen output was scarcely changed, being on the day of the experiment 6.13 gm. and on the day following 5.74 gm. The urea and uric acid output also remained little

changed. After this experiment the normal nitrogen output fell to 5.22 gm. as the average of seven days. The average urea output for the same days was 4.06 gm. Another experiment with the administration of 2 gm. of nitrogen in the form of urea was made. The nitrogen and urea output after this administration remained practically unchanged. Thus, on the low nitrogen intake there was a retention of nitrogen even when it was administered in the form of urea.

Experiments with an Intake of Ten Grams of Nitrogen.—On a diet of 10 gm. of nitrogen the average daily output for six days was 8.82 gm. For the same period the urea output was 7.51 gm. per day, or 85.2 per cent. of the total nitrogen. The uric acid output was 0.0869 gm. per day and the nitrogen of the purin bases was 0.062 gm. per day.

Urea Experiments.—An experiment with the administration of an additional 2 gm. of nitrogen in the form of urea was made on December 14. During the first twenty-four hours after the administration there was an excessive output of nitrogen of 1.15 gm., or 57 per cent. of the total excessive intake. There was, however, a protracted increase in the output for four days, so that in this experiment the total increase in the output exceeded the intake, although the diuresis produced by the substance was insignificant.

On December 21 another experiment was performed with the administration of 2 gm. of nitrogen in the form of urea. During the first twenty-four hours of the experiment 0.36 gm. of nitrogen was removed, or 18 per cent. of the intake. The following day there was an increase of the nitrogen output of 0.59 gm., or 30 per cent. of the intake, and on the third and fourth days there was still a slight increase. The total increased output for four days exceeded the normal by 59 per cent. of the excessive intake.

Experiments with an Intake of Thirteen Grams of Nitrogen.—The nitrogen intake was again raised to 13 gm. per day and the additional substances were given on four successive days. The output was then followed for about eight days after the beginning of the administration of the additional substances. The average output for five days was 12.20 gm. The first experiment began on January 19. For four successive days 2 gm. of nitrogen in the form of urea were administered. During the first twenty-four hours 1.41 gm. of excessive nitrogen were removed, or 70 per cent. of the intake. The following day again 1.40 gm. of nitrogen were removed. In course of eight days from the beginning of the experiment 8.94 gm. of nitrogen were removed, of which 6.68 gm. were in the form of urea, 0.0751 gm. in the form of uric acid, and 0.0074 gm. in the form of purin bases.

It is seen from these figures that the elimination of the excessive nitrogen was most favorable on this diet and, therefore, experiments were performed with nucleic acid and with uric acid. The nucleic acid experiments began on January 31, 1910.

Nucleic Acid Experiments.—Two gm. of nitrogen in the form of nucleic acid were given on four successive days and the nitrogen output was followed for eight days after the beginning of the experiment. In the first twenty-four hours there was eliminated 0.68 gm. of nitrogen in excess of the normal output, and each following day the excessive nitrogen output increased in value reach-

ing on the fifth day 2 gm. The total excessive output for eight days was 8.09 gm. of nitrogen of which 5.91 gm. were in the form of urea, 0.500 gm. in that of uric acid, and 0.415 gm. in the form of purin bases.

Uric Acid Experiments.—On February 15, experiments with uric acid were begun. The experiments lasted four days. On each day the patient received 2 gm. of nitrogen in the form of uric acid. During the first twenty-four hours the nitrogen output remained unchanged. In the second twenty-four hours, 0.82 gm. of excessive nitrogen was removed; on the following day 1.50 gm., and in the course of nine days 7.15 gm. of nitrogen were removed, of which 5.22 gm. were in the form of urea, 0.381 gm. in the form of uric acid.

On March 7, the second experiment with uric acid was performed. Two gm. of nitrogen in the form of uric acid were given daily on four successive days. During the first twenty-four hours 0.59 gm. of excessive nitrogen was removed, on the second day 1.34 gm., and on the fifth day the nitrogen output came down to normal. In the course of the five days the total excessive nitrogen output was 5.39 gm., of which 5.17 gm. were in the form of urea, and 0.187 gm. in the form of uric acid.

It is seen from these figures that on a high nitrogen intake the removal of excessive nitrogen is more complete than on a low protein intake. But it is not so complete as in health, so that even urea is in part retained in the organism. The nitrogen of nucleic acid and uric acid is removed at a slower rate than that of urea. This, of course, occurs also in health. Comparing the character of nitrogen elimination after the administration of nucleic acid and of uric acid one notes that after uric acid administration the nitrogen output proceeded at a lower rate of speed, and was less complete. This is observed also in health. The increase in the uric acid output was 6.2 per cent. of the total in the nucleic acid period, and 5.3 and 3.5 per cent. respectively in the two uric acid periods.

THIRD SERIES OF EXPERIMENTS.

This series lasted from November, 1910, to April, 1911. In this series of experiments it was again aimed to ascertain whether the quantity of nitrogen in the standard diet influenced the rate of elimination of excessive nitrogen added to the standard diet. The patient was again placed on a diet containing about 6 gm. of nitrogen. He came to a condition approaching nitrogenous equilibrium when the average daily output for five days was 6.29 gm. of nitrogen.

Urea Experiments.—On December 3 an experiment was begun with the administration of urea. Two gm. of nitrogen in the form of urea were given on three consecutive days. There was no increase in the nitrogen output during the first twenty-four hours. During the second twenty-four hours the increase of nitrogen output was 0.076 gm., and on the third day 0.072 gm. After this the output came down to normal. In December the daily nitrogen output on

the standard diet was lower, reaching an average of 5 gm. per day. On December 13 an experiment was begun with the administration of 2 gm. of urea per day on three consecutive days. The excessive output for the first day was 0.35 gm., the second day 1.30 gm., the third day 1.65 gm., and for five days following the beginning of the experiment 4.54 gm. of nitrogen were removed.

Again it follows from these observations that on a low nitrogen diet the elimination of the excessive nitrogen was very incomplete. Hence the diet was changed so as to contain 13 grams of nitrogen per day. On this diet the daily output was 10.50 grams of nitrogen per day.

On January 23 an experiment was performed with urea. Two gm. of urea were administered per day on three consecutive days. The excessive output during the first twenty-four hours was 1.38 gm., or 69 per cent. of the intake. The following day the output was 2.36 gm., and in five days after the beginning of the experiment the excessive nitrogen output was 6.31 gm.

It is seen from these figures that again on this occasion the increase in the nitrogen intake brought about a condition which permitted the nitrogen added in the form of urea to be removed more completely and in a shorter period of time. Experiments were then performed with hypoxanthine, inosine, guanosine, and uric acid.

Hypoxanthine Experiments.—1. Two gm. of nitrogen in the form of hypoxanthine were given on three successive days beginning January 30. On the first day of the experiment 1.02 gm. of excessive nitrogen were removed, and in five days following the beginning of the experiment there were removed 4.1 gm. of nitrogen in excess over the normal, or 69.5 per cent.

2. The second experiment with hypoxanthine was performed on February 13, when 2 gm. of nitrogen in the form of hypoxanthine were given daily for three days. During the first twenty-four hours there was an excessive elimination of 0.23 gm. of nitrogen, the second day 0.84 gm. of nitrogen, and the course of six days from the beginning of the experiment 4.47 gm. of nitrogen were removed, or 74.5 per cent. of the excessive intake.

The daily output of nitrogen on the standard diet changed again about this period, reaching 10.85 grams per day.

Inosine Experiments.—One gm. of nitrogen in the form of inosine was given on three consecutive days beginning February 24. The excessive nitrogen output on the first day of the experiment was 0.65 gm. of nitrogen, and in three days of the experiment there were removed 2 gm. of excessive nitrogen, or 60 per cent. of the excessive intake.

The nitrogen output on the standard diet changed again, giving an average of about 10.17 grams of nitrogen.

Uric Acid Experiments.—Two gm. of nitrogen in the form of uric acid were given on three consecutive days beginning March 6. The excessive output during the first twenty-four hours of the experiment was 0.81 gm., on the second day 1.31 gm., and in five days after the beginning of the experiment there were eliminated 6.43 gm. of excessive nitrogen, or 100 per cent. of the excessive intake.

After this period the condition of the patient was such that the physician deemed it advisable to prescribe diuretin for him. The daily output on the normal diet then rose to 11.96 grams per day.

Guanosine Experiments.—One and a half gm. of nitrogen in form of guanosine were given in two days beginning April 7. In the first twenty-four hours there was an excessive nitrogen output of 0.46 gm.; in three days after the beginning of the experiment there was an increase of nitrogen output of 2.36 gm., or 78.6 per cent. of the total intake.

In order to facilitate the analysis of the behavior of the different derivatives of nucleic acid after their administration the figures of the excessive nitrogen elimination are presented in the following tables (tables II, III, and IV).

GUANINEHEXOSIDE OBTAINED ON HYDROLYSIS OF THYMUS NUCLEIC ACID *

By P. A. LEVENE AND W. A. JACOBS.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

It has been established by the writers that in the plant nucleic acids, in the guanylic acid and in inosinic acid the purine is linked to the sugar in a glycosidic union. The direct proof was presented by the isolation of the pentosides corresponding to the purines: adenosine, guanosine, hypoxanthosine (inosine), uridine and cytidine. The assumption was made that in the thymo-nucleic acid the purine bases had the same mode of linking as in the other acids. Direct proof, however, was missing. Schittenhelm, London and Wiener¹ have thought that they obtained guanosine on digestion of thymo-nucleic acid by intestinal juice. The substance isolated by them gave the orcin test and had the appearance of guanosine. However, thymo-nucleic acid contains no pentose and therefore cannot yield guanosine (guanine riboside). Undoubtedly the nucleic acid employed by them was contaminated with guanylic acid. This consideration does not vitiate in any way the conclusion of those writers concerning the mechanism of nucleolysis, but leaves the problem of the structure of the thymo-nucleic acid where it was previous to their publication.

Following the discovery of the nucleosides contained in the inosinic, guanylic and nucleic acids of plant origin, repeated attempts were made to obtain the nucleosides composing the thymus nucleic acid. All methods employed successfully on the other occasion led to no results in the experiments with thymus nucleic acid. Also a great many modifications of the old methods resulted in complete failure. Hence it was concluded to resort to enzymes. After considerable search, an enzyme was selected which permitted the iso-

* Received for publication, July 19, 1912.

¹ *Zeitschr. f. physiol. Chem.*, lxvii, p. 459.

lation of one of the nucleosides entering into the structure of the molecule of the complex nucleic acid of animal origin. The substance obtained is guaninehexoside. It was semicrystalline, soluble in hot alcohol and separated from this solution on cooling. The substance has the composition $C_{11}H_{15}N_5O_6$. It did not reduce Fehling's solution and with silver nitrate formed a precipitate only when the solution was neutral. The silver compound immediately dissolved in a minimal excess of ammonia water. It did not give the orcin test on direct boiling with hydrochloric acid but gave the test with hydrochloric acid containing some copper. After hydrolysis the substance, on boiling with Fehling's solution, formed a voluminous white precipitate of cuprous guanine compound. From the products of hydrolysis were obtained an osazone melting at 198° C. and guanine sulphate.

EXPERIMENTAL PART.

The reaction mixture of nucleic acid and enzyme was made alkaline with ammonia and to the mixture 98 per cent alcohol was added as long as a precipitate formed. This was removed by filtration, and the mother liquor concentrated to dryness under diminished pressure. This was again dissolved in water, the solution rendered alkaline with an excess of ammonia and the treatment with alcohol repeated. The final filtrate was again concentrated to dryness under diminished pressure. The residue was taken up in water and the nucleotides still present in the solution removed by means of a solution of basic lead acetate. The precipitate thus formed was removed by filtration and to the filtrate ammonia and more lead acetate solution added as long as a precipitate formed. The last precipitate was filtered and washed carefully with cold water. It was finally suspended in water and decomposed by means of hydrogen sulphide. The filtrate from lead sulphide was rendered slightly alkaline by means of ammonia and evaporated under diminished pressure to a thick syrup. (It is very important not to omit this step, since the purine hexosides are easily decomposed even by acetic acid.) On cooling, the syrup is transformed into a gelatinous mass resembling crude guanosine. The substance was then filtered on a suction flask over silk and then dis-

solved in hot alcohol, filtered and allowed to cool. A sediment was formed on cooling, composed of rosettes resembling impure leucine.

The analysis of this substance gave the following data :

0.1120 gram of the substance gave 0.1746 gram of CO_2 and 0.0538 gram of H_2O .

0.1500 gram of the substance employed for a Kjeldahl nitrogen estimation required for neutralization 23.5 cc. of $\frac{N}{10}$ sulphuric acid.

	Calculated for $\text{C}_{11}\text{H}_{15}\text{N}_5\text{O}_6$	Found.
C	42.20	42.50
H	4.84	5.34
N	22.30	21.93

For hydrolysis about 0.200 gram of the substance was dissolved in 2 per cent sulphuric acid and heated on a water bath with return condenser for half an hour. The base was removed with silver oxide. Silver purine was filtered and decomposed with hydrochloric acid. The filtrate from the silver chloride was allowed to stand in the refrigerator and a sediment of guanine hydrochloride formed. The hydrochloride was transformed into the free base by means of ammonia and this again transformed into the sulphate.

The filtrate from the silver purine was freed from silver and the free sulphuric acid neutralized with sodium acetate. The clear solution served for the preparation of the phenylosazone. For purification it was recrystallized out of water containing very little pyridine. The osazone had the melting point of 198°C .

ON CEREBRONIC ACID.*

By P. A. LEVENE AND W. A. JACOBS.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Cerebronic acid was first discovered by Thudichum,¹ who erroneously considered it an isomer of stearic acid and named it neurostearic acid. It was the merit of Thierfelder to correct the view of the first discoverer and to demonstrate that the substance was an oxyacid of the composition $C_{25}H_{50}O_3$ and not $C_{18}H_{36}O_2$. In justice to Thudichum it must be stated that his analytical data agreed sufficiently with the figures required by the new formula for the substance. There is, however, a considerable disagreement in the data of the two observers in regard to the melting point of their substances, Thudichum recording the melting point at 84–85° C. and Thierfelder² at 98–99°. On the basis of this there was still open the possibility that the two substances had a different structure. Hence it was urgent to establish the relationship of the two substances.

Concerning the structure of cerebronic acid, it was established by Thierfelder that it contained one hydroxyl group; and it was also made very probable by him that the molecule contained twenty-five carbon atoms. It is well known that the percentage composition of carbon and hydrogen in the higher fatty acids does not furnish sufficient information regarding the number of carbon atoms in the molecule. With a greater degree of probability this can be established on the basis of the "acid value" of the substance.

Regarding the place of the hydroxyl group and regarding the character of the linking between the carbon atoms the work of Thierfelder offered no information. The work of this author also failed to take cognizance of the optical activity of the substance.

* Received for publication, July 19, 1912.

¹ Thudichum: *Die chemische Konstitution des Gehirns*, Tübingen, 1901, pp. 194, 195.

² *Zeitschr. f. physiol. Chem.*, xliii, p. 21, 1904.

The results of the present investigation led to the conclusion that cerebronic acid is the normal α -hydroxypentacosanic acid. In the hydrolysis mixture it occurs in the form of two isomers: the optically active dextrorotatory form of $[\alpha]_D^{20} = +4.16^\circ$ and the optically inactive form. The active substance melts at a point somewhat higher than given by Thierfelder,⁸ being $106-108^\circ \text{C.}$, the inactive at $82-85^\circ \text{C.}$ Thus, apparently, Thudichum had in his hand the optically inactive substance, while Thierfelder's was undoubtedly a mixture of the two. It is possible, of course, that a substance with still higher rotatory power may be obtained.

The place of the hydroxyl group was established by the fact that on oxidation with alkaline permanganate solution the cerebronic acid gave rise to an acid of the composition $\text{C}_{24}\text{H}_{48}\text{O}_2$.

The normal character of the carbon chain was made probable by the transformation of cerebronic acid into a hydrocarbon that melted between $54-57^\circ \text{C.}$ According to Kraft⁴ and Marie⁵ the three nearest hydrocarbons have the following melting points: $\text{C}_{24}\text{H}_{50}$, 51°C. ; $\text{C}_{25}\text{H}_{52}$, $53.5-54.0^\circ \text{C.}$; $\text{C}_{26}\text{H}_{54}$, 58°C.

Thus it seems very probable that the hydrocarbon obtained from cerebronic acid had the composition $\text{C}_{25}\text{H}_{52}$. This assumption is further substantiated by the fact that the acid value for the pure cerebronic acid corresponded to molecular weight value of $\text{C}_{25}\text{H}_{50}\text{O}_3$.

The optically active substance was separated from the inactive by fractional precipitation with lithium acetate.

EXPERIMENTAL PART.

Preparation of Cerebronic Acid.

The acid was obtained from cerebrine. Fifty grams of the cerebroside were taken up in 1,500 c.c. of methyl alcohol containing 70 grams of sulphuric acid to each 1,000 c.c. The mixture was heated with return condenser in a water bath for six hours and the separated acid and ester allowed to cool at 0°C. They were then filtered on suction over silk and boiled with an excess of alcoholic

² *Zeitschr. f. physiol. Chem.*, xliii, p. 21, 1904.

⁴ *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 1711, 1882.

⁵ *Bull. de la soc. chim.*, xv, p. 567, 1898.

soda in a water bath with return condenser for about four hours. The mixture was then again allowed to cool and the soaps filtered off. These were dissolved in glacial acetic acid containing enough hydrochloric acid to combine with the base of the soap. The solution was then poured into a large volume of distilled water from which the free acid separated. It was then filtered and dissolved in boiling alcohol, from which it separated on cooling.

Purification of the Crude Cerebronic Acid.

Several processes were used for this purpose, but none of them can as yet be recommended as final. Considerable effort was lost in an attempt to obtain an acid with a very sharp melting point. It was found that the acid melting at a higher temperature was less soluble in ether, petroleic ether (boiling at 60° C.) and ligroin (boiling at 80° C.). The purification was therefore accomplished by repeated recrystallizations of the substance from one of the three solvents as long as the melting point was rising. The final substance obtained in that manner had a melting point of 102° C., which, however, was not very sharp. This substance was further purified by fractionating it out of a methyl alcoholic solution with an alcoholic solution of lithium acetate. In this instance the acid obtained from the top fraction had a fairly sharp melting point at 106–108° C. The substance obtained from the lower fraction had a melting point of 82–84° C.

Ultimate Analysis of the Two Acids.

Not less than twenty different samples of different melting points were analyzed. They all agreed well with the theory and here will be given only three of them, the first melting at 106–108° C., the second at 95° C. and the third at 82–84° C.

- (1) 0.1224 gram of the substance gave 0.3388 gram CO₂ and 0.1360 gram H₂O.
- (2) 0.1206 gram of the substance gave 0.3342 gram CO₂ and 0.1356 gram H₂O.
- (3) 0.1194 gram of the substance gave 0.3292 gram CO₂ and 0.1350 gram H₂O.

	Calculated for C ₂₆ H ₅₀ O ₈ .	1	Found. 2	3
C	75.33	75.42	75.40	75.60
H	12.50	12.43	12.65	12.57

Optical Activity of the Acids.

Since the acids had the same percentage composition and differed in their melting points it was natural to look for the cause of it in possible stereoisomerism, since the acid contains a hydroxyl group in the molecule. As was stated already, the substance with the highest melting point had also the highest optical activity.

0.2190 gram of cerebronic acid (M.P. = 106° C.) dissolved in 5.0 cc. of pyridine. Total weight = 5.0126 grams; showed a rotation of +0.35° in pure yellow light and in a 2 dm. tube at 20° C.

$$[\alpha]_D^{20} = +4.01^\circ (\pm 0.00^\circ)$$

0.2229 gram of the same substance fractionated once more by means of lithium acetate was dissolved in 5 cc. of pyridine. Total weight = 5.0960 grams. Rotated in 2 dm. tube and in pure yellow light 0.37° to the right.

$$[\alpha]_D^{20} = +0.965^\circ (\pm 0.00^\circ)$$

0.2667 gram of cerebronic acid melting at 92° C. was dissolved in 5 cc. of pyridine. Total weight = 5.0763 grams. Rotation in 2 dm. tube in pure yellow light = +0.10°.

$$[\alpha]_D^{20} = +4.16^\circ (\pm 0.01^\circ)$$

0.2000 gram of cerebronic acid, melting at 82–84° C., dissolved in pyridine. Total weight = 5.0578 grams. Proved inactive.

Molecular Weight Estimation.

The molecular weight was determined by titration with an N/2 solution of sodium hydrate. The acid was dissolved in a mixture of benzol and pure methyl alcohol. It is not always an easy matter to obtain the acid of sufficient purity for the purpose. The easiest way of obtaining the substance is by way of the lead salt.

(1) 1.4394 grams of the acid dissolved in benzol + methyl alcohol. Titrated with N/2 alkali. Phenolphthalein used as indicator. Result calculated to N/10 value = 36.00 cc.

(2) 1.5896 grams of cerebronic acid treated in the same manner as the previous required 47.25 cc. of N/10 alkali.

(3) 1.3073 grams cerebronic acid treated as above required 32.2 cc. of N/10 alkali.

(4) 2.2440 grams cerebronic acid treated as above required 57.5 cc. of N/10 alkali.

(5) 1.5000 grams cerebronic acid required for titration 37.8 cc. of N/10 alkali.

	Calculated for $C_{25}H_{40}O_8$		Found.				
M. W.	398.0	399.0	397.0	406.0	396.8	397.0	

Determination of the Place of the Hydroxyl Group in the Cerebronic Acid.

This was determined by oxidation of cerebronic acid by an alkaline solution of potassium permanganate.⁶ Numerous experiments were performed, the conditions of oxidation remaining unchanged. They were the following:

Nine grams of analytically pure cerebronic acid were suspended in 300 c.c. of water containing 3 grams of potassium hydrate and heated until the acid formed a gelatinous soap. This was then added to a hot solution of potassium permanganate, made up of 1 liter of water and 4.5 grams of potassium permanganate. The heating was continued until the color of permanganate changed to a brownish tint. The boiling was then discontinued and the mixture decolorized by means of sodium bisulphite. The insoluble soaps generally collected on the surface of the fluid.

The subsequent treatment differed in individual experiments. In some the soaps were immediately transformed into the free acids, and this fractionated by means of ether and of ligroin. In other experiments it was attempted to first separate the salts on the basis of the differences of their solubility in alcohol.

The most convenient procedure is the following: Separate the sodium salts into two fractions, one soluble in hot alcohol, the other insoluble. The latter fraction consists principally of unchanged cerebronic acid. The hot alcoholic solution of soaps is allowed to cool until the soaps separate out. These are filtered and converted into the free acids. In this fraction the acid $C_{24}H_{48}O_2$ predominates. Its purification is based on the property of its lithium salt to remain insoluble in boiling methyl alcohol. The lithium salt is converted into the free acid. For final purification it was transformed into the lead salt and this again reconverted into the free acid.

Analysis of the Acid $C_{24}H_{48}O_2$.

(1) 0.1220 gram of the acid gave 0.3492 gram CO_2 and 0.1382 gram H_2O .

(2) 0.1238 gram of another sample gave 0.3568 gram CO_2 and 0.1400 gram H_2O .

0.1228 gram of the second sample gave 0.3550 gram CO_2 and 0.1428 gram H_2O .

* Edmed: *Journ. Chem. Soc.*, lxxiii, pp. 627-634, 1898.

- (3) 0.1214 gram of a third sample gave 0.3504 gram CO_2 and 0.1400 gram H_2O .
 0.1212 gram of the third sample gave 0.3480 gram CO_2 and 0.1400 gram H_2O .
 (4) 0.1212 gram of the substance gave 0.3470 gram CO_2 and 0.1446 gram H_2O .

	Calculated for $\text{C}_{25}\text{H}_{46}\text{O}_2$.		Found.		
		1	2	3	4
C	78.20	78.10	(a) 78.45 (b) 78.75	(a) 78.70 (b) 78.40	78.40
H	13.16	12.69	(a) 12.72 (b) 12.99	(a) 12.91 (b) 12.92	13.40

Analysis of the Sodium Salt of the New Acid.

0.1636 gram of the sodium salt gave 0.0300 gram Na_2SO_4 ; Na = 5.94 per cent.

	Calculated for $\text{C}_{25}\text{H}_{47}\text{O}_2\text{Na}$.	Found.
Na	5.90	5.94

Molecular Weight Estimation of the New Acid.

0.9500 gram of the acid dissolved in benzol and methyl alcohol and titrated with $N/2$ alkali; figures given in $N/10$ alkali. Required, 26.5 cc. of $N/10$ alkali.

0.9578 gram of the same substance required for neutralization 25.75 cc. of $N/10$ alkali.

	Calculated for $\text{C}_{25}\text{H}_{46}\text{O}_2$.	I Found.	II
M. W.	368.0	375.0	371.0

Melting Point of the New Acid.

The melting point of the new acid was at $81-82^\circ\text{C}$. Of the two known acids having the composition $\text{C}_{25}\text{H}_{46}\text{O}_2$, one, lignoceric, melts at 80.5°C . and the other, carnaubic, melts at 72.5° . It is possible that the substance is identical with lignoceric acid.

Reduction of Cerebronic Acid with Hydroiodic Acid.

The object of the first experiment under the conditions to be here described was to reduce the hydroxyl group and thus to obtain an acid of the composition $\text{C}_{25}\text{H}_{50}\text{O}_2$. Unexpectedly a substance was obtained containing considerable phosphorus, but the organic radicle of the substance possessed the composition of a hydrocarbon. The experiments were all repeated under identical conditions. It is possible that the yield might have been improved by following the directions of Kraft⁷ for the reduction of fatty acids to the corresponding hydrocarbons, but the simplicity of this process was very attractive.

Lots of 2 grams of the acid with 10 c.c. of hydroiodic acid, of

⁷ Ber. d. deutsch. chem. Gesellsch., xv, p. 1687, 1882.

specific gravity 1.96, and 0.5 gram of red phosphorus were heated in sealed tubes at 125° C. for four hours. The tubes were then opened, the hydroiodic acid diluted with an equal volume of water, again sealed and heated at 110–115° C. for about three hours. The product of reaction had a white solid appearance. It was washed with water, dissolved in hot alcohol and allowed to cool. The substance solidified and was filtered. The substance was again dissolved in alcohol, a few drops of phenolphthalein added and rendered alkaline by means of an alcoholic solution of sodium hydrate. The solution was evaporated to dryness. The dry mass was transferred to a flask and distilled at about 0.5 mm. pressure. The distillate was purified by redissolving in hot alcohol and allowing the solution to cool. The substance was then filtered and freed from adhering alcohol by melting.

Many samples were analyzed. The analysis of two will be reported here.

0.1300 gram of the substance gave 0.4092 gram of CO₂ and 0.1673 gram of H₂O.

0.1041 gram of the substance gave 0.3277 gram of CO₂ and 0.1337 gram of H₂O.

	Calculated for C ₂₅ H ₅₂	I	Found. II
C	85.10	85.30	85.70
H	14.90	14.31	14.37

The Melting Point of the Hydrocarbon.

The melting point of one sample of the hydrocarbon was 53–56° C., of another, 54–57° C. Kraft determined the melting point of the normal tetracosan at 51.1°, of heptacosan 59.5° C., and Marie found for pentacosan 53.5–54° C. Perhaps if we had sufficient material for a second distillation of the paraffin the melting point might have come down to that of Marie. On the other hand, the difference in the melting points of normal, tri- and tetracosan is 3.4° C., and according to this the melting point for pentacosan may be expected to be 54.5° C., against 53–56° C. found for our substance.

ON THE CEREBROSIDES OF THE BRAIN TISSUE.*

By P. A. LEVENE AND W. A. JACOBS.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Under the general name of cerebrosides have been described a number of individual substances designated by special names and apparently possessing distinct chemical and physical properties. The exact relationship of the substances one to another has not been fully established. The subject has been treated in a critical way in several exhaustive articles written by men as competent as Frankel,¹ Leathes,² Bang³ and Cramer.⁴ One is surprised to find that the views of these writers are in disagreement on some of the fundamental points regarding the relationship of different cerebrosides. The better known substances of this group are phrenosine and kerasine of Thudichum;⁵ cerebrine, homocerebrine and encephaline. The first two of the latter three were studied by Parcus⁶ and by Kossel and Freytag.⁷ Finally to the same group belong cerebrin of Thierfelder and pseudocerebrine of Gamgee. Bang, the most skeptical of the writers, believes in the existence of only two cerebrosides: phrenosine and kerasine. Cramer and Leathes are inclined to distinguish between cerebrine and cerebrin, and admit the individuality of kerasine and of homocerebrine. The latter two substances they consider identical. Also Frankel is inclined to regard cerebrine, cerebrin, pseudocerebrine and phrenosine as one substance. On the basis of personal experience this view was also defended by Gies and Posner.⁸

* Received for publication, July 19, 1912.

¹ *Ergeb. d. Physiol.*, viii, p. 212, 1909.

² *The Fats*, Longman, Green and Co., 1910.

³ *Chemie und Biochemie der Lipide*, Wiesbaden, 1911.

⁴ *Biochemisches Handlexicon*, iii, p. 225, 1911.

⁵ *Die chemische Konstitution des Gehirns*, 1901.

⁶ *Journ. f. prakt. Chem.*, xxiv, p. 310, 1881.

⁷ *Zeitschr. f. physiol. Chem.*, xvii, p. 431, 1893.

⁸ *This Journal*, i, p. 59, 1905.

However, it must be remarked that the views of most of the writers are not supported by indisputable experimental proof. The hypotheses are mostly a matter of personal preference, and most writers express them with great caution. The difficulty in co-ordinating the data on the different cerebrosides is largely due to the fact that the investigations have been carried out by different workers at very distant intervals and no one worker attempted to co-ordinate his results with those of his predecessors. It was realized that three principal cerebrosides must be closely related one to another, but it was not clear wherein the point of distinction was contained.

It was the aim of the investigation, here to be presented, to search for these.

As there existed complete accord regarding the identity of the base and of the carbohydrate entering into the composition of phrenosine, cerebrine, cerebron and kerasine, their point of distinction has to be looked for in the fatty acids that helped to make up their molecules. In reality the data regarding the melting points and the percentage composition of the fatty acids present striking differences. Hence our attention was directed to the study of the fatty acids of the mixed cerebrosides as obtained by the process of Parcus. At first it seemed as though an acid could be isolated that was richer in carbon than cerebronic acid and which had a very low melting point. But on purification of that substance it always showed the composition of cerebronic acid, although it always possessed a low melting point. It was then discovered that the acid with the low melting point was the optically inactive cerebronic acid. Thus no evidence was obtained in favor of the view that another acid than cerebronic acid was present among the decomposition products of the mixed cerebrosides. If it was present its quantity undoubtedly was very small. Hence, the cerebrosides referred to in this publication seem to be identical from the standpoint of the character of their components.

Another fact supporting the view of the chemical identity of the cerebrosides was found in the following:

The mixed cerebrosides were fractionated first according to Parcus on the basis of the difference in their solubility in alcohol.

Each fraction was then recrystallized from glacial acetic acid. Under this condition from cerebrine and from kersine substances were obtained that possessed the same composition and the same melting point as cerebron and hence, could be both regarded as cerebrine or cerebron. In the glacial acetic acid mother liquor there remained a small quantity of substance that was extremely soluble in glacial acetic acid and in alcohol at room temperature. The proportion of this substance was very small and as yet has not been further studied. However, the fact was made clear that the major part of cerebrine and of kersine was composed of the substance having the composition of cerebron. And yet, when entirely free from impurities, the different fractions differed in their solubility in alcohol and in their physical appearance as they settled out of alcohol. The differences were the same as described by Parcus for cerebrine and homocerebrine.

The similarity of cerebrine, cerebron, and kersine is also supported by the bromine estimation made by Kossel and Freytag. There is only one double bond in the molecule of cerebron, which could lead to the formation of a dibrom derivative, and not a tribrom body as assumed by Kossel and Freytag. The theory for a dibrom cerebron requires 16.22 per cent. of bromine, whereas Kossel and Freytag found 16.60 and 16.30 per cent. for cerebrine and 16.90 and 17.25 per cent. for kersine.

It is also noteworthy that in all the three cerebrosides the primary amino group of the sphingosine is substituted, as none of them, treated with nitrous acid in the apparatus of Van Slyke, gives rise to nitrogen gas.

A difference appeared only when the optical activity of the substance was studied. It was found generally that the substances obtained from the cerebrine fraction were dextrorotatory, while those obtained from the kersine fraction were generally optically inactive, and once a sample was obtained from the kersine fraction that was strongly levorotatory. Furthermore, by means of a mixture of pyridine and alcohol it was found possible to fractionate the dextrorotatory substance into a fraction practically inactive and into another with a dextrorotation higher than that of the original substance.

Since the three cerebroside are distinguished principally by differences in solubility and since there is no evidence to the effect that they are composed of different elements, one may feel justified in advancing the hypothesis that the three cerebroside are all mixtures of stereoisomeric substances. The difference in the solubility of the various substances may be determined by the proportion of the optically active and inactive substances. It would perhaps be simpler to abandon most of the nomenclature and to refer to cerebroside as *d*-cerebrine, *l*-cerebrine and *dl*-cerebrine. Of course, it still is necessary to discover a method by which a separation could be accomplished conveniently. Cerebrine, cerebrin and phrenosine correspond to *d*-cerebrine; kerasine and homocerebrine to *dl*-cerebrine. Of course the possibility is not excluded that the lipoids of the brain contain cerebroside of an entirely different order.

EXPERIMENTAL PART.

Preparation of Cerebroside.

Part of the cerebroside were prepared following the directions of Parcus, the other part by a slight modification of the process. The details were the following: Brains freed from the membranes were passed through a hashing machine and suspended in a barium hydrate solution. The mixture was brought to a boil and the solid brain tissue separated from the liquid by filtering through cheese cloth. The residue was then suspended in 95 per cent. alcohol and the mixture was brought to a boil in a percolator. The alcohol was then removed and the extraction continued with 95 per cent. alcohol containing 5 per cent. of ammonia water. The extraction was continued as long as the alcohol gave a precipitate on cooling. The combined alcoholic extracts were placed in a refrigerator at -1° C. A sediment formed on standing. This was again dissolved in 95 per cent. alcohol and again placed in the refrigerator. The precipitate of mixed cerebroside thus formed was then extracted by means of absolute alcohol in a water bath at 65° C. The alcoholic filtrate was placed in a thermostat and there allowed to cool. The precipitate thus formed was considered as cerebrine in the sense of Parcus. In the early phases of the work

the mother liquors from cerebrine were allowed to stand at room temperature over night, which gave rise to an intermediate fraction; the mother liquor from this was placed in the refrigerator, and the precipitate there formed was regarded as homocerebrine or kerasine. Later the intermediate stage was omitted and the mother liquor from cerebrine placed immediately in the refrigerator. The traces of ash still present were removed according to the directions of Parcus.

Proportion of the Three Fractions in the Mixed Cerebrosides.

About 35 grams of the mixed cerebrosides dissolved in absolute alcohol were allowed to stand in the thermostat room over night; the precipitate, thus formed, filtered and dried in a vacuum desiccator, weighed 16.5 grams or 47.4 per cent. From the mother liquor of this at room temperature separated out 4 grams or 11.4 per cent. of the second fraction, and finally, from the second mother liquor separated in the refrigerator at -1° , 14.2 grams of the third precipitate or 40.6 per cent. of the total cerebrosides.

Precipitate I = 47.4 per cent. of total.

Precipitate II = 11.4 per cent. of total.

Precipitate III = 40.6 per cent. of total.

Purification of the Fractions.

(a) *By Means of Glacial Acetic Acid.*—It was then found that every one of these fractions could be fractionated by means of glacial acetic acid into two fractions, the soluble and insoluble. The soluble fractions were freed from the acid by distillation under diminished pressure. The residue was redissolved in hot alcohol and on cooling gave a precipitate; this again could be fractionated by means of glacial acetic acid, so that finally the greatest part of the cerebrosides could be crystallized out of glacial acetic acid.

(b) *By Means of a Mixture of One Part of Pyridine to One Part of Alcohol (98 per cent.).*—The precipitate obtained from the glacial acetic acid can be fractionated by this mixture. One part of the substance was dissolved in thirty parts of the mixture and allowed to stand over night at room temperature. The filtrate on cooling in an ice and alcohol mixture formed a second precipitate which was filtered off. The mother liquor from the second precipi-

tate, evaporated to dryness at diminished pressure and recrystallized out of alcohol, gave a very small third precipitate. The fractionation by means of this solution was very sharp with the substance obtained from the cerebrine fraction. When the substance obtained from kersine was dissolved in the pyridine mixture the physical appearance of the two fractions was not very different one from the other, and the precipitate filtered with difficulty.

Analysis of the Substances Obtained from the Various Fractions.

Cerebrine Out of Alcohol.

(1a) 0.1359 gram of the substance gave 0.3442 gram of CO_2 and 0.1388 gram H_2O .

(1b) 0.1304 gram of the substance gave 0.3324 gram of CO_2 and 0.1326 gram H_2O .

(1a) 0.1920 gram of the cerebrine out of alcohol was employed for a Kjeldahl nitrogen estimation. It required for neutralization 2.5 c.c. of $\text{N}/10$ acid. $\text{N} = 1.9$ per cent.

(1b) 0.2154 gram of cerebrine out of alcohol employed for a Kjeldahl nitrogen estimation required for neutralization 2.6 c.c. of $\text{N}/10$ acid. $\text{N} = 1.69$ per cent.

Cerebrine Out of Glacial Acetic Acid.

(2a) 0.1210 gram of the substance gave 0.3092 gram of CO_2 and 0.1188 gram H_2O .

(2b) 0.1180 gram of the substance gave 0.2986 gram of CO_2 and 0.1152 gram H_2O .

Kersine Out of Alcohol.

(1) 0.1198 gram gave on combustion 0.3078 gram of CO_2 and 0.1258 gram of H_2O .

(1) 0.1524 gram of the same substance employed for Kjeldahl nitrogen estimation required for neutralization 1.7 c.c. of $\text{N}/10$ acid; $\text{N} = 1.60$ per cent.

Kersine Out of Glacial Acetic Acid.

(2a) 0.1132 gram of the substance gave on combustion 0.2872 gram CO_2 and 0.1168 gram H_2O .

(2b) 0.1102 gram of the substance gave on combustion 0.2836 gram CO_2 and 0.1188 gram H_2O .

	C	H	N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Calculated for cerebrin, $\text{C}_{48}\text{H}_{98}\text{NO}_9$	69.65	11.24	1.70
Found for cerebrine {	1a.	11.43	1.90
	1b.	11.37	1.69
	2a.	10.96	
	2b.	10.92	
Found for kersine { (homocerebrine)	1a.	11.75	1.60
	2a.	11.55	
	2b.	12.06	

The Melting Points of the Various Cerebrosides.

Regarding the melting points of the cerebrosides the records of individual investigators show marked variations. The lowest value found by Parcus and by Kossel and Freytag for kersine was 155–156° C., and the highest was recorded by Wörner and Thierfelder for cerebrin, at 209–212° C. The melting points of our substances also varied, depending on the degree of purification of the substances. The cerebrosides crystallized out of alcohol and, still containing some ash, showed decomposition rather than melting at the temperature of about 200° C. Substances carefully purified and, particularly, those purified by recrystallization out of glacial acetic acid possessed a very sharp melting point of 195° C. On fractionation out of the pyridine mixture two fractions were obtained: a more insoluble, melting at 210° C., and a less insoluble, melting at 195–198° C. The character of melting, described by Wörner and Thierfelder for their cerebrin, leads us to the belief that the sample in their possession undoubtedly consisted of a mixture of the isomers.

Optical Rotation of the Various Fractions.

Two samples were employed for the determination of the optical activity of the cerebrosides: one of the cerebrine and one of the kersine (homocerebrine) fractions. They had been recrystallized from glacial acetic acid, and the analytical data from them have been reported in this communication. They had a sharp melting point, first liquefying and then decomposing at 195° C. In macroscopic appearance the first substance had the character of a fine granular powder, the other that of a solid waxy mass.

The optical rotation of the fractions was the following:

Cerebrine.

0.5295 gram of the substance was dissolved in 5 c.c. of pyridine. Total weight = 5.3249 grams and rotation at $t = 25^\circ$ C. and in pure yellow light in 1 dm. tube was 0.10° (± 0.00) to the right.

$$[\alpha]_D^{25} = +1.01^\circ.$$

Kerasine.

0.8175 gram of the substance was dissolved in 8 c.c. of pyridine. Total weight = 8.3755 grams. There was no optical activity detectible in a 1 dm. tube and in pure yellow light.

Rotation of the Substances Fractionated by a Pyridine Alcohol Mixture.

Five grams of each of the tested substances were dissolved in 30 c.c. of warm pyridine to which 120 c.c. of 98 per cent. alcohol had been added. The solution was allowed to stand at room temperature (25° C.) over night, then filtered on suction, washed with ether and dried. The mother liquor was chilled in an alcohol ice mixture and filtered at low temperature. The rotations of the fractions were the following.

Cerebrine.

First fraction: 0.5090 gram of the substance dissolved in about 10 c.c. of pyridine. Total weight = 9.4852 grams. Rotation at 25° C. in pure yellow light and in 2 dm. tube = + 0.20°.

$$[\alpha]_D^{25} = + 1.88^\circ (\pm 0.00^\circ).$$

Out of 5 grams of the original substance about 2.5 grams of this first fraction and about 0.5 gram of a second fraction separated on cooling. The second fraction was inactive. The mother liquor from the second fraction gave a residue very soluble in alcohol. This was not tested further.

Kerasine.

Originally inactive. The first fraction, or the one insoluble at room temperature, was optically tested. 0.4988 gram of the substance was dissolved in 10 c.c. of pyridine. Total weight = 9.4282 grams. Rotation in 2 dm. tube, at room temperature of 25° C., and in pure yellow light = + 0.05° (± 0.00°).

$$[\alpha]_D^{25} = + 0.47^\circ (\pm 0.00^\circ).$$

The second fraction from the same sample was inactive.

The values of the specific rotation of all these substances were lower than those reported by Thierfelder for cerebrin, hence one is led to the conclusion that they consisted of mixtures of isomers.

Hydrolysis of Cerebrine for the Purpose of Estimating the Proportion of Galactose.

Experiment 1.—Twenty grams of cerebrine were taken up in 500 c.c. of alcohol containing 50 grams of sulphuric acid and boiled in a water bath with return condenser for six hours. Preliminary experiments have shown that the maximal hydrolysis cannot be obtained in a shorter time. The solution was then placed in the re-

frigerator over night. The fatty acids and esters were then removed by filtration. The mother liquor was then diluted with one liter of water and boiled with return condenser five hours in a water bath. The product of hydrolysis was then freed from sphingosine and from alcohol and the sugar estimated by reducing Fehling's solution, estimating the cuprous oxide by Volhard's method. The yield of galactose was 5 grams or 20 per cent.

Experiment 2.—Three grams of cerebrine were taken up in 50 c.c. of alcohol containing 5 grams of sulphuric acid and treated in the same manner as in the preceding experiment. The yield of galactose was 0.56 gram or 18.7 per cent.

Theory for cerebrin, $C_{48}H_{88}NO_8$, required 21.5 per cent. of $C_6H_{12}O_6$.

Found: Experiment 1.....20.0 per cent.

Experiment 2.....18.7 per cent.

Taking into consideration that the removal of the fatty acid and of sphingosine always leads to some loss the agreement is quite satisfactory.

Analysis of the Fatty Acids.

The mixture of fatty acids and esters obtained on hydrolysis of the mixed cerebrosides was saponified in the usual way and the soaps transformed into the free acids. These were separated into two fractions by means of ether. The fatty acids were dissolved in boiling ether and the ethereal solution allowed to stand in the refrigerator. A fraction separated consisting practically of pure cerebronic acid. The first precipitate thus obtained often had the composition of C=76.4, 76.3 or 76.1, and H=12.6, 12.3 or 12.4. A comparatively small part of the total acids always remained in the ethereal mother liquor. The acids were obtained from this by removing the ether by distillation and recrystallizing the residue out of alcohol or out of acetone. The precipitate thus obtained varied in its carbon content from 77 to 78.5 per cent., and hydrogen content of about 12.5 to 13.0 per cent. It was thought that an acid of the composition $C_{24}H_{48}O_2$ may be present in that fraction. However, in every experiment the acid value was low, and on the other hand, the fraction contained no methyl or ethyl esters, since

the presence of methyl or ethyl groups could not be demonstrated by the method of Zeist and Fanto or by the saponification value.

In the later phases of the work it was learned that cerebronic acid formed a lithium salt, soluble in hot alcohol, and the same salt of $C_{24}H_{48}O_2$ was insoluble. When the fraction containing the high carbon value was transformed into the lithium salt it was found that it was either all soluble in hot methyl alcohol or, if some remained as an apparently insoluble residue, it also was composed principally of cerebronic acid, containing carbon 76.5 per cent. and hydrogen about 12.7 per cent. Thus the attempts to identify the acid $C_{24}H_{48}O_2$ among the products of hydrolysis of mixed cerebrosides were not successful.

An experiment was then made in which 30 grams of cerebrine purified by glacial acetic acid were hydrolyzed in the usual way and the free acids were transformed into the lead salt and this again freed from lead. The analysis of the acid obtained in this manner had the following composition.

0.1194 gram of the substance gave 0.3292 gram CO_2 and 0.1350 gram H_2O .

	Calculated for $C_{28}H_{50}O_8$ (Cerebronic acid).	Found.
C	75.38	75.40
H	12.56	12.65

Thus the attempts failed to discover on hydrolysis of mixed cerebrosides an acid that was not cerebronic acid. If such is present it is undoubtedly in the form of an impurity derived from some substance other than the cerebrosides.

THE AMINO-ACID NITROGEN OF THE BLOOD. PRELIMINARY EXPERIMENTS ON PROTEIN ASSIMILATION.*

By DONALD D. VAN SLYKE AND GUSTAV M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Two opposing views are held concerning the manner in which the organism assimilates the amino-acids formed in the intestine from digested proteins.

Abderhalden¹ believes that in passing the intestinal wall the amino-acids are synthesized into a blood protein. This enters the circulation, from which the cells of the tissues take it, break it down again into amino-acids and from these rebuild their own characteristic proteins. The reason for this view, that the amino-acids are resynthesized into protein while passing the intestinal wall, is, that although amino-acids are abundant in the intestinal contents, attempts to demonstrate them in normal blood have met with absolute failure. Against this view the objection is valid, that it is based on negative results and that more sensitive methods than those hitherto applied might reveal amino-acids in the blood.

Buglia² offers evidence indicating that the body is capable of utilizing amino-acids which have entered the blood stream. He injected intravenously into dogs amounts of amino-acid mixtures comparable to those formed from the food, and found that, when the injection was performed slowly, no serious ill effects followed and only a small part of the amino-acid nitrogen was excreted. Folin and Denis³ injected large amounts of amino-acids directly into the small intestines of cats and noted a subsequent increase in the fraction of blood nitrogen left after subtracting the protein

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¹ *Synthese der Zellbausteine*, 1912.

² *Zeitschr. f. Biol.*, lviii, p. 162, 1912. Buglia also gives earlier literature.

³ This *Journal*, xi, p. 87, 1912.

and urea. Neither the results of Buglia nor those of Folin and Denis, however, demonstrate the presence of amino-acids in the normal blood during periods of either digestive activity or rest. So long as this demonstration remains unperformed, the contention of Abderhalden cannot be refuted, that during normal digestion, with the chyme gradually passing in small portions from the stomach to the intestine and from the intestine to the blood, the latter passage is accompanied by synthesis of food amino-acids into blood protein.

It was evident that for the solution of the problem a method was necessary sufficiently delicate to ascertain decisively whether amino-acids are present in or absent from the blood, and sufficiently accurate to detect the fluctuations normally occurring in amino-acid nitrogen if it is present. We have found that the nitrous acid method for determination of amino nitrogen can be easily used for the blood and answers the above requirements. We have consequently applied it to the problem of protein assimilation.

METHOD FOR DETERMINATION OF AMINO-ACID NITROGEN IN BLOOD.

Thirty to fifty cubic centimeters of freshly drawn blood are mixed with 9 or 10 volumes of 95 per cent alcohol to precipitate the proteins. The volume of the alcohol-blood mixture must be known; but in case it is not convenient to use a graduated cylinder for the mixture, its volume can be taken as the sum of the volumes of alcohol and blood without essentially affecting the results. The alcohol and blood are thoroughly mixed, the vessel containing them is closed and twenty-four hours are allowed for precipitation of the proteins to become complete. The solution is filtered through a dry folded filter into a measuring cylinder, without washing the precipitate. The volume of filtrate obtained is noted, and is taken for analysis as an aliquot part of the total blood-alcohol mixture. The filtrate is then concentrated to a volume of 3–5 cc. and used for determination of amino nitrogen by Van Slyke's nitrous acid method.⁴ The use of a few drops of acrylic alcohol to prevent foaming is advisable.

The precipitation with alcohol is a simple and effective means of removing the proteins. The filtrate, even when concentrated, gives no biuret test. The completeness of the removal of pro-

⁴ This *Journal*, ix, p. 185; xii, p. 275.

teins is further evidenced by the fact that when in control tests the alcohol was driven off from the filtrate and the residue was hydrolyzed with hydrochloric acid, the amino nitrogen set free by the hydrolysis amounted to only 2 mgm. per 100 cc. of blood.

Whether the alcoholic filtrate is concentrated on the water bath or under diminished pressure appears to make little difference with the results. We have, however, as a regular thing, concentrated under diminished pressure in a double-necked flask, transferring the solution towards the end of the distillation to a small flask, from which it can be removed with a minimum amount of wash water. This method of concentration is the most rapid, and the lipoids which separate when the alcohol is driven off take the form of a fine emulsion, which does not interfere mechanically or otherwise with the amino determination. They can be removed with ether, but this is unnecessary.

For the amino determination one can either wash the concentrated filtrate into a 10 cc. graduated flask, measuring off 9.7 or 9.8 cc. of this in the burette of the amino apparatus; or one can transfer the filtrate directly from the small distilling flask into the 10 cc. burette of the amino apparatus, using several portions of 2-3 cc. of water each to clean out the flask and wash the solution completely into the deaminizing bulb. We have usually used the latter method.

The amino determination is much simpler than in the case of urine, because the amount of ammonia in the blood is negligible, and the urea content is so slight that the correction for the proportion of urea nitrogen (about 3 per cent), given off while the amino-acids are quantitatively decomposed, is scarcely sufficient to affect the significance of results if it were entirely neglected. We have, however, always determined and made this correction according to the principle utilized in the determination of amino nitrogen in the urine.⁵ The correction rests on the fact that in two to four minutes (according to the temperature) amino-acids give off 100 per cent of their nitrogen. Continuing the reaction for an equal length of time thereafter results, when urea is present, in the evolution of a further amount of nitrogen from the urea, this amount being equal

⁵ *This Journal*, xii, p. 275.

to that evolved from the *urea* while the amino-acid was being decomposed in the first period. With the small amounts of urea present in the blood it is unnecessary to make an entire extra analysis to determine the correction. The latter is ascertained as follows. For the decomposition of the amino-acids the reaction is run with constant rapid shaking for four minutes at a temperature below 20° , for three and a half minutes at $20-25^{\circ}$ and for two to three minutes above 25° , the time being accurately measured. At the end of the reaction the nitrogen gas is purified and measured in the usual way, and then expelled from the apparatus through cock *c* (see figure 2, p. 278, preceding number of this *Journal*). The solution left in the deaminizing bulb *D* is now shaken and treated exactly as described on page 280 of the above article for determining whether the reaction is complete or not. The length of time between the end of the first reaction and that of the second should be accurately that of the first reaction. The gas formed during the second period is shaken out with permanganate and measured. This represents the urea correction and is to be deducted from the first reading. The latter, minus this correction, represents only amino-acid nitrogen, since other forms of amino nitrogen, such as fatty amines, amino-purines, amino-pyrimidines and ammonia, react slowly and would if present be corrected for with the urea. In case it is inconvenient to finish the second reaction in as short a period as the first, it may be allowed to run one or two minutes longer, the correction being calculated on the rate of urea nitrogen evolved per minute. It is essential that the solution should be shaken rapidly during the last minute to expel all the nitrogen formed. The fact that under these conditions very nearly the same amount of nitrogen is evolved in the first and second periods is indicated by the following four pairs of determinations, each of which was made with 10 cc. of 0.5 per cent urea.

	I. cc.	II. cc.	III. cc.	IV. cc.
N in first three minutes	2.0	2.2	2.1	2.4
N in second three minutes	2.2	2.2	2.2	2.6

The maximum difference, 0.2 cc., is equivalent to only 0.1 mgm. of amino nitrogen; and the corrections actually found in blood work are usually less than 2 cc.

To ascertain whether the technique above described involves losses of amino-acids, particularly whether they are precipitated with or adsorbed by the proteins, the following experiment was performed.

One hundred cc. of fresh defibrinated blood, drawn from a dog which had fasted twenty-four hours, was precipitated with 1 liter of alcohol. (Total volume = 1100 cc.) Of the filtrate, 800 cc., equivalent to 72.7 cc. of blood, were concentrated, freed from lipoids with ether and brought to 25 cc. Ten cc. portions, equivalent to 29.1 cc. of blood, were used for amino determinations.

I.	cc.	II	IV. cc.
N in first 4 minutes.....	2.95	N in first 3½ minutes.....	2.85
N in second 4 minutes.....	0.60	N in second 3½ minutes.....	0.50
N from amino-acids.....	2.35		2.35

2.35 cc. of nitrogen at 26°, 754 mm., from 29.1 cc. of blood indicate 4.42 mgm. of amino-acid nitrogen per 100 cc.

To another 100 cc. of the same blood 1 gram of alanine, much more amino-acid than we have ever encountered in the blood in nature, was added. Other details of the analysis were identical with the above.

	I.	II.
N gas in three and one-half minutes	85.2 cc.	85.6 cc.
N from amino-acids and urea of blood.....	2.9 cc.	2.9 cc.
N from added alanine.....	82.3 cc.	82.7 cc.
Temperature	26°	27°
Pressure	754 mm.	754 mm.
Alanine N per 100 cc. found	0.1554 gm.	0.1550 gm.
Alanine per 100 cc. found	0.9880 gm.	0.9860 gm.
Alanine per 100 cc. found	1.0000 gm.	1.0000 gm.

AMINO-ACID CONTENT OF BLOOD FROM NORMAL FASTING DOGS.

The animals used for the following experiments had fasted for twenty to twenty-four hours before the blood was drawn.⁶ The results indicate that the amino-acid content varies within relatively narrow limits.

⁶For the samples from the carotid artery and the vena cava we thank Dr. Auer and Dr. Githens.

DOG NO.	SOURCE OF BLOOD	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N (CORRECTED FOR UREA)	TEMPERATURE	PRESSURE	AMINO-ACID NITROGEN PER 100 CC. BLOOD
		cc.	cc.	degrees C.	mm.	mgm.
1	Femoral artery ...	29.1	2.35	26	754	4.4
1	Femoral artery ...	29.1	2.35	26	754	4.4
2	Femoral artery ...	28.4	2.10	25	762	4.1
2	Femoral artery ...	28.4	2.20	25	762	4.3
3	Vena cava	21.5	2.10	24	760	5.4
4	Carotid artery....	59.0	5.80	25	760	5.4
5	Femoral artery ...	51.0	4.20	25	760	3.7
6	Femoral artery ...	35.5	3.30	25	760	5.2
7	Carotid artery....	37.5	2.20	31	758	3.1
8	Femoral artery ...	40.0	3.00	30	758	4.0
8	Mesenteric vein...	40.0	2.90	30	758	3.9

Experiment I. Behavior of Intravenously Injected Alanine.

A male dog of 14 kilos weight was catheterized and the bladder was thoroughly washed out. One cannula was placed in the right femoral vein, another in the left femoral artery. A sample of blood was drawn from the artery, and immediately afterwards the injection of 12 grams of *dl*-alanine dissolved in 400 cc. of water into the vein of the other leg was commenced. At intervals samples of blood were drawn. At the end of the experiment the bladder was again washed out and the excreted amino-acid nitrogen determined. The results are here summarized.

	TIME	TIME AFTER END OF INJECTION	FREE AMINO-ACID N PER 100 CC.	ESTIMATED ALANINE IN CIRCULATION	UNPRECIPITATED AMINO NITROGEN PER 100 CC. FREED BY HYDROLYSIS
	p. m.	minutes	mgm.	grams	mgm.
Drew blood sample I. Normal..	3:15		4.2	0.00	2.1
Began injection.....	3:17				
Finished injection.....	3:30				
Drew sample II.....	3:35	5	37.2	1.47	1.8
Drew sample III.....	3:42	12	20.8	0.64	1.5
Drew sample IV.....	4:05	35	12.3	0.36	1.4
Drew sample V.....	4:30	60	12.3	0.36	1.7

Total nitrogen in urine0.445 gram.

Ammonia nitrogen in urine.....0.015 gram.

Free amino-acid nitrogen in urine.....0.236 gram.
equivalent to 1.501 grams alanine.

Total amino-acid nitrogen in urine.....0.233 gram.

The excreted alanine was optically inactive.

The injected alanine disappeared from the blood with such rapidity that five minutes after the injection only 1.47 grams out of the 12 grams injected remained in circulation and in thirty-five minutes only 0.36 gram. A relatively small proportion, 1.5 grams in all, was excreted, about 10 of the 12 grams *being almost immediately taken from the circulation by the tissues*. There was no evidence of synthetic processes in the blood, for the amino nitrogen freed by hydrolysis, which might be expected to increase if intermediate products of protein synthesis were present, remained practically constant.

The total amount of alanine in circulation was calculated on the assumption that the volume of the blood in liters is one-twentieth of the weight of the body in kilograms. While this is, of course, only a rough approximation, it suffices for the purpose of the experiment.

The details of the analyses follow :

*Experiment I. Determination of Free Amino-Acid Nitrogen. Temperature 25°;
Pressure 762 mm.*

SAMPLE	VOL. OF SAMPLE	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS EVOLVED IN 4 MIN.	N GAS IN FOLLOWING 4 MIN.	N GAS (CORR.) FROM AMINO-ACIDS	AMINO-ACID N PER 100 CC. BLOOD
	cc.	cc.	cc.	cc.	cc.	mgm.
I	{ 87	28.4	2.3	0.2	2.1	4.1
		28.4	2.4	0.2	2.2	4.3
II	{ 100	31.6	22.1	0.9	21.2	37.2
		24.0	9.3	0.4	8.9	20.7
III	{ 76	24.0	9.4	0.4	9.0	20.9
		29.2	6.8	0.4	6.4	12.1
IV	{ 91	29.2	7.0	0.4	6.6	12.5
		24.8	5.9	0.4	5.5	12.3
V	{ 75	24.8	5.8	0.3	5.5	12.3

For the above determinations each filtrate was brought to 25 cc., duplicate determinations being made on 10 cc. portions of this.

Of the unused parts, 4 cc. portions were used for hydrolysis. They were mixed with equal volumes of concentrated hydrochloric acid and heated at 100° for twenty-four hours. The acid was evaporated off as completely as possible, and the ammonia removed by vacuum distillation with calcium hydrate. The latter was then dissolved with acetic acid and the sample used for amino determination.

*Experiment I. Determinations of Amino Nitrogen After Hydrolysis.
Temperature 27°; Pressure 764 mm.*

SAMPLE	VOL. OF BLOOD EQUIVALENT TO SAMPLE USED	N GAS IN 3 MIN.	N GAS IN FOLLOWING 3 MIN.	N GAS FROM AMINO- ACIDS	AMINO N PER 100 CC. BLOOD	FREE AMINO N	AMINO N FREED BY HYDROLYSIS
	cc.	cc.	cc.	cc.	mgm.	mgm.	mgm.
I	11.4	1.50	0.10	1.40	6.3	4.2	2.1
II	12.6	9.10	0.20	8.90	39.0	37.2	1.8
III	9.6	4.50	0.20	4.30	22.3	20.8	1.5
IV	11.7	3.05	0.15	2.90	13.7	12.3	1.4
V	9.9	2.65	0.15	2.50	14.0	12.3	1.7

The urine voided during the hour after the beginning of the injection was made up to 100 cc. Portions of 2 cc. used for Kjeldahl determinations required 6.40 and 6.30 cc. of N/10 acid, the average indicating 0.445 gram of total nitrogen.

Fifty cubic centimeters were used for determination of ammonia and free amino nitrogen, as described by Levene and Van Slyke.¹ The ammonia neutralized 5.2 cc. of N/10 HCl, indicating 0.0146 gram of ammonia N in the entire urine. The ammonia-free urine was brought back to 50 cc. volume and 10 cc. portions used for determination of free amino-acid nitrogen.

I.	cc.	II.	cc.
N in first 3 minutes.....	45.0	N in first 4 minutes.....	42.2
N in second 3 minutes.....	1.9	N in second 4 minutes.....	2.2
Amino-acid N.....	43.1	Amino-acid N.....	43.0

These determinations were made at 27°, 758 mm. The average, 43.05 cc. indicates 0.236 gram of free amino-acid nitrogen in the total urine. This includes all the amino nitrogen, comparison with the following determination showing that no measurable amounts were conjugated.

For determination of the total amino nitrogen 40 cc. of urine were used, the sample being diluted to 50 cc. after removal of urea and ammonia. Ten cc. portions were used for amino determinations. They gave 33.5 and 33.4 cc. of nitrogen at 25°, 762 mm. indicating 0.233 gram of total amino nitrogen in the urine.

In order to determine whether the organism had destroyed or retained the natural component of the amino-acid (*d*-alanine) and excreted the other (*l*-alanine), as found by Wohlgemuth⁸ to be the case when certain *dl*-amino-acids were given *per os*, 25 cc. of ammonia-free urine, containing 0.375 gram of alanine, were concentrated, and acidified with 0.5 cc. of concentrated hydrochloric acid. The weight of the solution was 4.273 grams, the concentra-

¹ This *Journal*, xii, p. 275.

⁸ *Ber. d. deutsch. chem. Gesellsch.*, xxxviii, p. 2064, 1904.

tion of alanine hydrochloride 12.3 per cent. This, if all *l*-alanine, should have given a rotation of -1.2° in a 1 dm. tube. The observed rotation was only -0.04° . The *dl*-alanine was, therefore, excreted practically unchanged and the alanine retained was equal parts dextro and levo.

Experiment II. Absorption of Alanine from the Small Intestine.

The animal used was a dog of 10 kilos weight which had fasted for twenty-four hours. Cannulas were placed in the left femoral artery and the mesenteric vein. Fifty cc. samples of blood were drawn from each and diluted to 500 cc. with alcohol. A loop of the small intestine was then ligated at both ends and 15 grams of alanine, dissolved in 100 cc. of water, injected into it. After forty minutes, samples of blood were again drawn. The loop was then washed out, its contents diluted to 500 cc., and 10 cc. used to determine the unabsorbed alanine nitrogen. The determination yielded 26 cc. of nitrogen at 26° , 760 mm. From this:

	Grams.
Total alanine N injected.....	2.461
Alanine N unabsorbed	0.718
Alanine N absorbed.....	1.743

The blood analyses gave the following results. The temperature was 30° , barometer 758 mm.

SOURCE	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS EVOLVED IN 2.5 MIN.	N GAS IN FOLLOWING 2.5 MIN.	N GAS FROM AMINO- ACIDS	AMINO-ACID N PER 100 CC. BLOOD
	cc.	cc.	cc.	cc.	mgm.
Femoral artery before in- jection*.....	40	3.7	0.7	3.0	4.0
Mesenteric vein before in- jection.....	40	3.7	0.8	2.9	3.9
Mesenteric vein after injection.	43	5.6	0.6	5.0	6.3

* The determination on the blood from the femoral artery after the injection was lost.

The amount of amino-acid nitrogen in the mesenteric blood increased by 60 per cent as the result of the injection of alanine into the intestine. This, however, demonstrates only the possibility that amino-acids can pass the intestine. The sudden flooding of the intestine with a solution of one or more amino-acids is so entirely

different from the gradual entrance of partially digested proteins from the stomach which occurs in normal digestion, that the results can not be utilized to explain the normal process of protein assimilation. The same restriction applies to the results of Folin and Denis,⁹ who flooded the intestines of cats with solutions containing unusual amounts of amino-acids and observed a subsequent rise in the fraction of blood nitrogen left after subtracting the urea and protein.

THE RISE OF THE AMINO-ACID CONTENT OF THE BLOOD DURING DIGESTION.

Experiment III.

We have, therefore, performed the following experiment. Samples of 50 cc. of blood were drawn from the right femoral arteries of two dogs, of about 15 kilos weight each, which had fasted for

Experiment III. Effect of Digestion on the Amino-Acid Content of the Blood.

SOURCE OF BLOOD	VOL. OF BLOOD EQUIVALENT TO FILTRATE USED	N GAS IN 3.5 MIN.	N GAS IN FOLLOWING 3.5 MIN.	N GAS FROM AMINO- ACIDS	AMINO-ACID N PER 100 CC. BLOOD
	cc.	cc.	cc.	cc.	mgm.
<i>Dog A</i>					
Right femoral artery before feeding.....	51.0	4.6	0.4	4.2	3.7
Mesenteric vein after feeding..	38.3	8.3	1.5	6.8	9.5
Left femoral artery after feeding.....	37.7	7.2	1.2	6.0	8.6
<i>Dog B</i>					
Right femoral artery before feeding.....	35.5	4.0	0.7	3.3	5.2
Mesenteric vein after feeding..	38.5	9.4	2.3	7.4	10.2
Left femoral artery after feeding.....	37.3	9.1	2.2	6.9	9.9

twenty-four hours. The dogs were in normal condition the next day, and each then devoured a kilo of fresh beef. Five hours after the meal the animals were etherized and samples of blood drawn from both the left femoral artery and the mesenteric vein.

The amino-acid content is about doubled during digestion. It will be noticed that the correction for urea also increases. This is to be expected from the results of Folin and Denis, who noted

⁹ This *Journal*, xi, p. 87, 1912.

a marked rise in the urea content of the blood during digestion of protein.

SUMMARY OF RESULTS.

The gasometric method for direct determination of amino-acid nitrogen is easily applicable to blood from which the proteins have been precipitated by alcohol. Duplicate results usually agree within 0.2 mgm. of amino-acid nitrogen per 100 cc. of blood.

The blood of the dog normally contains amino-acid nitrogen. The amount, in animals which have been fasting for twenty to twenty-four hours, is 3 to 5 mgms. per 100 cc. of blood.

Twelve grams of alanine, injected during 13 minutes into the vein of a dog, were so rapidly removed from the blood stream that five minutes after the injection only 1.5 grams were left in the blood, and after 35 minutes but 0.4 gram. Only 1.5 grams were excreted in the urine, the greater part of the injected amino-acid being evidently taken up by some of the tissues.

Absorption of 10 grams of alanine from the small intestine increased the amino-acid nitrogen of the mesenteric blood from 3.9 to 6.3 mgm. per 100 cc.

During normal digestion of meat the amino-acid content of the blood undergoes a marked increase compared with its value before feeding. It was doubled in the case of one dog and somewhat more than doubled in that of another. The increase affected the blood from the femoral artery almost as much as that directly from the mesenteric vein.

CONCLUSIONS.

With the finding of amino-acid nitrogen in the normal blood the hypothesis, that the amino-acids formed in digestion are synthesized into blood protein while passing the intestinal wall, becomes superfluous. The increase in amino-acid nitrogen of the blood, noted during digestion of protein, is furthermore, positive evidence that amino-acids as such do normally pass the intestinal wall and enter directly into the blood current. The fact that the amino-acid content decreases but little during passage of the blood from the mesenteric vein out to the femoral artery indicates that the

amino-acids are not held back or destroyed by the liver before reaching the other tissues. On the contrary, it seems that the amino-acids absorbed from the intestine circulate through the entire organism and are offered directly to the body cells in general. The fact that the amount of amino-acids normally present in the circulation is small is accounted for by the rapidity with which the tissues take up amino-acids from the blood as soon as they become unusually abundant therein. This is illustrated by the disappearance of intravenously injected alanine from the blood stream.

The experiments here reported are preliminary to a more complete investigation of the problem of protein assimilation and of the effect of different physiological and pathological conditions upon the amino-acids of the blood.

ON THE STRUCTURE OF THYMUS NUCLEIC ACID.*

BY P. A. LEVENE AND W. A. JACOBS.

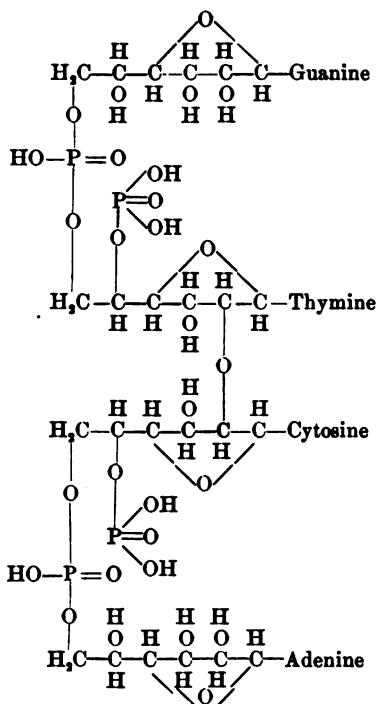
(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Following the successful characterization of the inosinic and guanylic acids as phosphoric acid esters of purine ribosides, *i. e.*, simple nucleotides, and of the yeast nucleic acid as a complex of four such nucleotides, we at once attempted the same with the thymus nucleic acid. Evidence had already been obtained by Levene and Mandel¹ of the probable existence of a thymine nucleotide in this acid but owing to the meager means at hand at that time for studying the product obtained the result required confirmation. From the very start our progress was greatly retarded by the marked differences shown by this acid towards the chemical treatment to which the ribose nucleotides had so readily yielded. The nucleic acid, when heated with ammonia in a sealed tube under the conditions for the preparation of the yeast nucleosides, remained apparently unchanged. When the temperature was raised to a point at which phosphoric acid was cleaved, free purines were also obtained. Likewise, when subjected to "neutral hydrolysis" the cleavage of phosphoric acid was always accompanied by the appearance of free purines and even pyrimidines. From the mixture no nucleosides could be isolated. This was finally accomplished by other methods which will be described elsewhere. We have been led to conclude that the instability of the sugar is the cause of the failure of chemical methods. Whereas the ribose nucleotides on hydrolysis with acids yield strongly reducing mixtures, the thymus nucleic acid shows but the weakest reduction of Fehling's solution after hydrolysis. That portion of the sugar which has been cleaved from combination is converted at once into levulinic acid. This was accomplished by boiling only two hours with 2 per cent sul-

* Received for publication, July 19, 1912.

¹ *Ber. d. deutsch. chem. Gesellsch.*, xli, p. 1905, 1908.

phuric acid. Levene² has already pointed out that the glucosidic linking in this acid is much weaker than that in the yeast nucleic acid, as shown by the ready cleavage of all purines in the former by boiling in dilute acetic acid.



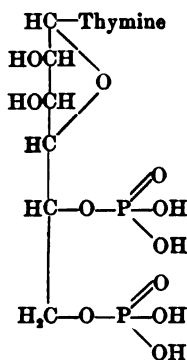
On the other hand the union of the phosphoric acid in the thymus nucleic acid resembles in stability that of the inosinic acid. Whereas by heating with dilute ammonia the phosphoric acid is most readily removed from the yeast and guanylic acids, under the same conditions both the inosinic and thymus nucleic acids remain unchanged. The inosinic acid is decomposed only by neutral hydrolysis, a condition under which unfortunately the glucosides of the thymus are destroyed. This stability, together with the stability of the pyrimidine glucosides which is analogous in every respect to the conditions found in the yeast nucleic acid, has enabled us to obtain by partial hydrolysis with dilute acid intermediary products

² *Biochem. Zeitschr.*, x, p. 215, 1908.

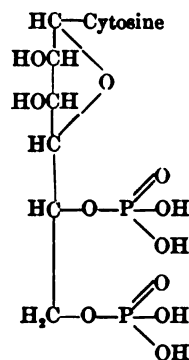
from which we have been able to construct a formula representing in all probability the structure of the acid.

It has been assumed by many that the nucleus of the nucleic acid is a polyphosphoric acid in which the phosphoric acid molecules are linked together in an anhydride form. As is well known, the acid itself is stable towards alkali, a property not possessed by anhydrides, so that *a priori* such a structure is unlikely. As a matter of fact, the products formed upon hydrolysis of the acid are incompatible with such a mode of linking. As developed by the present investigation, the nucleic acid is represented by the formula on preceding page.

The two pyrimidine nucleotides are joined together presumably by an ether linking between the sugars. Each sugar of the thymine and cytosine nucleosides is conjugated with a secondary phosphoric acid and then again with a tertiary phosphoric acid which in turn forms a bridge between the pyrimidine and the purine nu-



Hexo-thymidine diphosphoric acid.



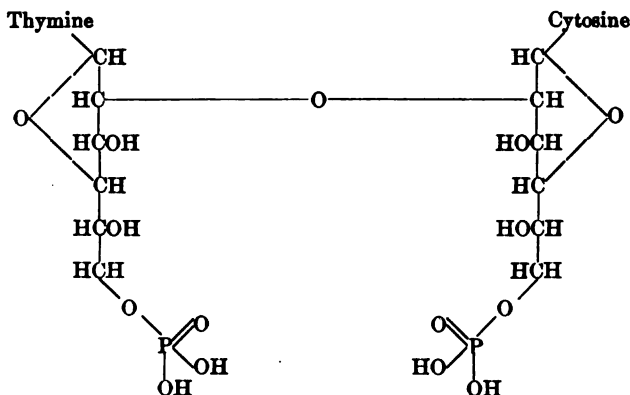
Hexo-cytidine diphosphoric acid.

cleosides. This view is based upon the following facts. Upon hydrolysis of the nucleic acid with 2 per cent sulphuric acid for two hours the purines are completely removed and the sugar originally in union with them is almost completely converted into levulinic acid. This was readily obtained by extraction with ether and identified by the hydrazid and silver salt. The resulting mixture, as described below, was fractionated with phosphotungstic acid. From the portion not precipitated by this reagent the crystalline brucine salts and barium salts of a hexo-cytidine diphosphoric acid and

a hexo-thymidine diphosphoric acid were obtained of the structure given on preceding page.

Though not yet proven, we consider this position of the phosphoric acids on the sugars probable, because of the great stability shown by them; a condition analogous to that found in inosinic acid.

From the phosphotungstic fraction we have succeeded in isolating a dinucleotide of the following structure.



In all these formulae no significance is to be attached to the configuration of the sugar since its nature has not yet been determined.

The crystalline brucine salt and barium salt of this substance were also prepared and from the barium content shown by the analysis we must conclude that each phosphoric acid contains a secondary and a tertiary hydroxyl and the linking between the two nucleotides occurs between the sugars as shown above. By fractionation with mercury a mixture of the barium salts of the simple thymine and cytosine nucleotides was obtained. The experimental part regarding both the dinucleotide and the simple nucleotides will be described in a subsequent communication.

EXPERIMENTAL PART.

Hydrolysis of the Nucleic Acid.

Two hundred grams of nucleic acid (from fish sperm) were heated on the water bath in 3 liters of 2 per cent sulphuric acid until

solution was complete and then boiled for two hours under a reflux condenser. After cooling silver oxide was added as long as a precipitate of purine silver separated and the mixture was allowed to stand over night. The filtrate from the purine precipitate was then treated further with silver oxide until a drop of the solution added to dilute sodium hydrate gave a precipitate of silver oxide. To this solution a warm saturated solution of barium hydrate was added until just alkaline to litmus. The precipitate which contained the silver and barium compounds of the nucleotides, phosphoric acid and free pyrimidines, was rapidly filtered by suction and washed well with water. The filtrate after removal of silver with hydrogen sulphide gave a considerable test with orcin and copper, and slightly reduced Fehling's solution. After removal of barium from this with sulphuric acid and concentration to a small bulk we were able to precipitate with basic lead acetate and barium hydrate a small amount of material from which a barium salt was prepared. This salt reduced Fehling's solution and contained conjugated phosphoric acid. The salt so obtained is presumably the barium salt of the hexose phosphoric acid contained in the nucleic acid. The quantity of substance which was still quite impure was too small to purify further.

The above silver barium precipitate was decomposed by suspending in a slight excess of dilute sulphuric acid and passing hydrogen sulphide through the mixture. After complete decomposition the filtrate was freed from hydrogen sulphide and precipitated by mercuric sulphate solution. The heavy precipitate of the mercury salt of the mixed nucleotides was filtered and well washed. In the filtrate are contained besides free phosphoric acid the simple nucleotides which were isolated by a method to be described later. The above mercury precipitate was suspended in water and completely decomposed by hydrogen sulphide. The mixture was neutralized with pure barium carbonate and then acidified with acetic acid to keep in solution some of the nucleotides. The filtrate, after concentration to a small volume, was precipitated by several volumes of alcohol. The heavy precipitate of barium salts was filtered, washed with 70 per cent alcohol, then with absolute alcohol and ether and then dried. The yield varied from 50 to 75 grams. The mixture

so obtained was dissolved in about 5–10 parts water and the barium removed with a slight excess of sulphuric acid. To the filtrate 50 per cent sulphuric acid was added until the solution contained 10 per cent acid. To this concentrated phosphotungstic acid was added until precipitation was complete. The phosphotungstate fell as a heavy gummy mass to the bottom and after standing a while at 20° the clear supernatant liquor was poured off. The phosphotungstate was again dissolved in several volumes of hot water and again precipitated by addition of sulphuric acid to 10 per cent. The phosphotungstate contained the dinucleotide fraction. The two mother liquors were joined and the phosphotungstic acid shaken out with amyl alcohol. The solution was then treated with barium hydrate solution until just alkaline to phenolphthalein, again acidified with acetic acid, filtered and concentrated to several hundred cubic centimeters. For further purification the nucleotides were precipitated again with mercuric sulphate and the mercuric precipitate decomposed with hydrogen sulphide. From the filtrate, after removal of the hydrogen sulphide, a trace of sulphuric acid was removed quantitatively with barium hydrate. The acidity of the solution was then determined by titration and an equivalent amount of commercial brucine dissolved in alcohol was added to the mixture. Though crystallization may begin at once, the whole mixture was concentrated *in vacuo* to dryness and the residue dissolved in a sufficient quantity of hot 85 per cent alcohol. On cooling the mixture crystallized to a solid mass. After standing twenty-four hours it was broken up with a glass rod, filtered and washed with 75 per cent alcohol. The brucine salt so obtained is the salt of the hexo-thymidine diphosphoric acid. After several recrystallizations from hot 85 per cent alcohol it is pure.

Hexo-Thymidine Diphosphoric Acid.

As so prepared the neutral or tetrabasic salt crystallizes in variegated aggregates of long microscopic plates. When rapidly heated in a capillary tube it sinters at about 172°. The acid salts crystallize in globular aggregates of microscopic needles of higher melting point. The neutral salt is practically insoluble in cold water, absolute alcohol, acetone, chloroform and the other neutral organic sol-

vents. In hot dilute alcohol and hot dilute acetone it is fairly easily soluble.

For analysis it was dried in vacuum over phosphorus pentoxide at 110° .

0.3358 gram substance gave 0.0375 gram $\text{Mg}_2\text{P}_2\text{O}_7$.

	Calculated for $\text{C}_{11}\text{H}_{18}\text{N}_2\text{O}_{12}\text{P}_3 \cdot 4\text{C}_{22}\text{H}_{38}\text{N}_2\text{O}_4$	Found.
P	3.06	3.11

For conversion into the barium salt the brucine salt was suspended in water in a separatory funnel, an excess of ammonia added and shaken out several times with chloroform. The aqueous solution of the ammonium salt was placed in a distilling flask, a few drops of phenolphthalein added, and distilled *in vacuo*, the receiving flask containing dilute sulphuric acid. During the distillation a saturated solution of pure barium hydrate was added drop by drop until the solution remained on continued distillation just alkaline to the phenolphthalein. After the removal of the ammonia by this process the barium salt, which had already partly separated upon the first addition of barium hydrate, was brought just into solution by the addition of a few drops of acetic acid, an excess being avoided. The solution was then brought to a boil. The barium salt which is more insoluble in hot than in cold water separated as a white powder, which under the microscope appeared as globules in which crystal formation was hard to discern. The salt was filtered hot, washed with hot water, then with alcohol and ether and dried. So prepared the salt is pure for analysis. For the analysis it was dried *in vacuo* over phosphorus pentoxide at 110° . Many samples obtained in different experiments were analyzed.

0.1879 gram substance gave 0.1248 gram CO_2 ; 0.0360 gram H_2O .

0.1579 gram substance gave 0.0514 gram $\text{Mg}_2\text{P}_2\text{O}_7$.

0.1512 gram substance gave 0.0990 gram BaSO_4 .

0.1397 gram substance gave 0.00574 gram N (Kjeldahl).

0.2425 gram substance gave 0.0101 gram N (Kjeldahl).

	Calculated for $\text{C}_{11}\text{H}_{14}\text{O}_{12}\text{N}_2\text{P}_3\text{Ba}_2$	Found.	
C	18.44	18.12	
H	1.96	2.13	
		I	II
N	3.91	4.11	4.18
P	8.66		9.06
Ba	38.30		38.54

For the optical determination 0.346 gram substance was dissolved in 5 cc. of N/5 HCl. Total weight of solution, 5.533 grams. In 2 dm. tube at 30° with D-light it rotated 0.84° to the right. Calculating the specific rotation of the free acid without regard to the specific gravity,

$$[\alpha]_D^{30} = +10.86^\circ.$$

When the strong acetic acid solution of this salt is concentrated slowly on the water bath it gradually separates as a white crust which under the microscope is seen to consist of aggregates of colorless needles. When exposed, the tertiary salt absorbs carbon dioxide from the air.

The free acid was not prepared crystalline. The aqueous solution of the free acid is precipitated by mercury salts, except the chloride, by ammonium molybdate and lead acetate as well as by other heavy metals. Phosphotungstic acid precipitates it as an oil in concentrated solutions when sulphuric acid is added to 20–25 per cent. This precipitate is soluble in water and precipitated by the addition of strong sulphuric acid. In following the method given above for the removal of the phosphotungstate fraction containing the dinucleotide, the sulphuric acid content of the mixture must not exceed 10 per cent to avoid precipitation of this thymine complex as well as the cytosine complex described below.

Upon hydrolysis of this acid with 10 per cent sulphuric acid in a sealed tube at 125° for three hours the phosphorus is completely broken off. From the mixture thymine was obtained in typical form and also levulinic acid.

With orcin and a drop of copper chloride solution the characteristic test for hexoses is obtained, but the green color is of considerably less intensity than that obtained with a hexose or with the original nucleic acid. This is in harmony with the experience obtained with the pyrimidine ribosides obtained from the yeast nucleic acid. As seen in the method of preparation the union of the pyrimidine with the sugar is comparatively stable, and no reducing substances are obtained upon hydrolysis. Also bromine water added to the complex is instantly decolorized and the resulting solution reduces Fehling's solution. Under certain conditions hydrogen with colloidal palladium was found to reduce the compound. The

resulting solution gave a very strong orcin test and upon hydrolysis yielded a strongly reducing solution containing a phospho-sugar and a dehydro-thymine. From these facts which agree with the experience obtained by Levene and Laforge⁸ with cytidine and uridine from the yeast nucleic acid we conclude that the union between pyrimidine and sugar is of the same nature in the two nucleic acids, *i. e.*, a simple glucosidic union.

Hexo-Cytidine Diphosphoric Acid.

The mother liquors obtained from the above brucine salt when concentrated to small volume yield, on standing, more of the salt of the thymine complex. When this is filtered and the mother liquors further concentrated and treated with absolute alcohol to permanent turbidity, on standing several days, the neutral brucine salt of the analogous cytosine complex separates in long plates with pointed ends which can be easily seen by the naked eye. After filtration the salt is dissolved in hot 95 per cent alcohol. On standing it separates as large prisms. After twenty-four hours the crystallization is complete. This salt is more beautiful than the thymine salt. It is likewise much more soluble in warm alcohol, even in hot absolute alcohol in which the thymine complex is very little soluble.

For the analysis it was again recrystallized and dried *in vacuo* over phosphorus pentoxide at 110°.

0.2839 gram substance gave 0.0363 gram $Mg_2P_2O_7$.

0.4878 gram substance gave 33.2 c.c. N (29°, 767 mm.).

	Calculated for $C_{10}H_{17}N_7O_{12}P_2 \cdot 4C_{21}H_{26}N_4O_4$	Found.
P	3.09	3.56
N	7.66	7.46

For the preparation of the barium salt the method described above for the thymine complex was used. It is also more insoluble in hot water than in cold water and advantage was taken of this fact for the preparation of the pure neutral salt. For analysis it was dried *in vacuo* over phosphorus pentoxide at 110°.

0.1922 gram substance gave 0.1203 gram CO_2 ; 0.0430 gram H_2O .

0.1484 gram substance gave 0.0085 gram N (Kjeldahl).

⁸ *Ber. d. deutsch. chem. Gesellsch.*, xlv, pp. 608-20.

0.1877 gram substance gave 0.0107 gram N (Kjeldahl).
 0.3681 gram substance gave 5.25 c.c. amino N (22.5°, 752 mm.).
 0.1730 gram substance gave 0.1162 gram BaSO₄.
 0.1507 gram substance gave 0.0493 gram Mg₂P₂O₇.

	Calculated for C ₁₀ H ₁₈ N ₂ O ₁₂ P ₂ Ba ₂ .	Found.
C	17.08	17.06
H	1.85	2.49
N	5.96	5.71
Amino N	1.99	1.59
P	8.82	9.11
Ba	39.00	39.55

The salt was obtained only as an amorphous powder. For the optical determination 0.3793 gram dried substance was dissolved in 4 cc. N hydrochloric acid solution. Total weight of solution, 4.427 grams. In a 2 dm. tube with D-light it rotated at 25°, 3.29° to the right. Calculating for the free acid and without regard for the specific gravity,

$$[\alpha]_D^{25} = + 31.45^\circ.$$

The free acid resembled closely in its behavior that of the thymine complex toward precipitants and reagents. It is likewise not more easily precipitated by phosphotungstic acid. The difference in solubility of the brucine salts is the only method of which we now know which will accomplish a separation of the two acids.

From this acid levulinic acid and cytosine were obtained. The latter was analyzed as the picrate.

0.1403 gram substance gave 30.9 c.c. N (27°, 764 mm.).

	Calculated for C ₄ H ₈ N ₂ O · C ₆ H ₃ (NO ₂) ₃ OH.	Found.
N	24.62	24.32

ON GUANYLIC ACID.

SECOND PAPER.*¹

By P. A. LEVENE AND W. A. JACOBS.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Guanylic acid undoubtedly belongs to the group of simpler substances of that class. Its molecule contains only one base, guanine, and one carbohydrate, *d*-ribose. These two substances are combined through a glycosidic linking as guanosine. There is, however, still a lack of agreement in the minds of individual investigators as to the degree of its simplicity. The latest publication on the subject is that by Bang² in 1910. In this article the author attacks with great ardor and considerable bitterness the view of those workers who regard guanylic acid as a simple nucleotide. According to Bang, guanylic acid in its structure is analogous to thymus nucleic and not to inosinic acid. The very lengthy argument of the author is based on the results of the ultimate analysis of various amorphous salts of guanylic acid and on the property of the salts to gelatinize. However, guanosine—a simple guanine-pentoside—shares with guanylic acid the property of gelatinizing when it contains only a slight proportion of mineral impurity. Also the argument of the elementary analysis is not very convincing for the reason that in the publication of Bang no evidence was offered to prove the purity of the analyzed acids. It is true that the opposing investigators have not yet succeeded in presenting analytical data of such a nature as to render their contentions absolutely convincing.

Several years ago the method of preparation of the acid was considerably simplified by us and we had hoped to be in a position to obtain the substance in that degree of purity which would permit us to clear up the disputed points in the structure of the substance.

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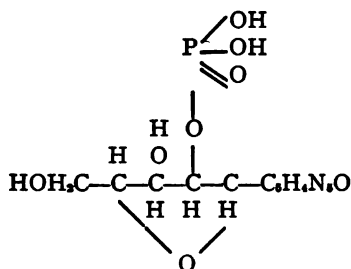
¹ *Ber. d. deutsch. chem. Gesellsch.*, xlii, p. 2469, 1909.

² *Biochem. Zeitschr.*, xxvi, p. 293, 1910.

Pressure of other work caused the delay of the undertaking and only very recently have we been in a position to again direct our efforts to the work on guanylic acid.

It is now possible to obtain the substance in the form of a pure crystalline brucine salt. The process that led to the preparation of this salt was complex. The acid was originally obtained directly from pancreas glands as an impure lead salt. This was transformed into an acid sodium salt. The latter salt was further purified as a mercury salt. The advantage of the last purification lay in the fact that a solution of mercuric sulphate forms a mercuric salt of guanylic acid insoluble in dilute acids. Thus a means is given by which all other bases may be removed from the mercuric salt. A solution of the free acid can be easily obtained from the mercuric salt. In that condition guanylic acid showed no tendency to gelatinize. The acid in this solution was easily transformed into the crystalline brucine salt and was analyzed as such. The brucine salt was then transformed into the neutral barium salt and this again analyzed. The analysis of the two last named salts permitted us to formulate the composition of guanylic acid as $C_{10}H_{14}N_5O_8P$ or as a mononucleotide. As a polynucleotide is formed through the process of anhydride formation, the polynucleotide hypothesis of the structure of guanylic acid requires values of carbon, nitrogen and phosphorus higher than those actually found.

On the basis of this the following constitution may be ascribed to guanylic acid:



The substance is optically active, showing $[\alpha]_D^{20} = -1.27^\circ$ in hydrochloric acid solution.

There is some basis for the assumption that the structure of guanylic acid is not identical with that of inosinic acid. There

apparently exists a difference in the union between phosphoric acid and the carbohydrate in these acids analogous to the difference in the union between phosphoric acid and the nucleosides of the two more complex acids, namely, that of the thymus and of the yeast nucleic acid. Both guanylic and yeast nucleic acid permit the detaching of phosphoric acid quite readily, whereas in the inosinic and in the thymus nucleic acid the same reaction is accomplished with great difficulty. This difference is probably due to a difference in the position of the phosphoric acid on the sugar. When hydrolyzed under the same conditions employed for the preparation of ribose phosphoric acid from inosinic acid, the phosphoric acid was completely cleaved from guanylic acid. We have, therefore, as yet been unable to obtain definite evidence regarding the position of the phosphoric acid on the sugar.

PREPARATION OF THE CRUDE GUANYLIC ACID.

The glands were trimmed and passed through a hashing machine. They were suspended in water, which was brought to a boil and to the mixture was added potassium acetate in substance enough to make the concentration 5 per cent.

When the mixture cooled down to about 65° enough sodium hydrate was added to make its concentration 5 per cent. This mixture was allowed to stand over night, when it was neutralized by means of picric and acetic acids as long as a precipitate formed. The precipitate was then removed by filtration and to the filtrate a 25 per cent solution of neutral lead acetate was added. The mixture was brought to a boil and filtered. To the filtrate more lead acetate and ammonia were added as long as a precipitate formed. This precipitate contained guanylic acid and guanosine. To obtain the guanylic acid the substance was suspended in hot water and transferred to flasks immersed in a hot water bath. A stream of hydrogen sulphide was passed through the mixture. The lead sulphide was ultimately removed by filtration and the filtrate concentrated to small volume. It was then rendered alkaline by means of ammonia and precipitated with a large excess of 95 per cent alcohol. This was done in order to remove the guanosine which remained in the solution, the mixed ammonium and sodium

salts of guanylic acid remaining in the precipitate. The precipitate was then dissolved in hot water and placed in the refrigerator where the acid salt of guanylic acid settled out as a gelatinous mass which was filterable without much difficulty.

In the early part of the work attempts were made to obtain crystalline sodium salts of this substance. The experiments were in a way successful but were later abandoned when a more convenient method for purification of the crude guanylic acid was devised.

PURIFICATION OF THE CRUDE GUANYLIC ACID.

The crude guanylic acid was suspended in water and sulphuric acid added until solution was complete. A solution of mercuric sulphate was then added as long as a precipitate formed. This was filtered and washed well with hot water. The precipitate was well suspended in water and completely decomposed by hydrogen sulphide. The mercuric sulphide was filtered off and a slight trace of free sulphuric acid in the filtrate removed quantitatively with barium hydrate. The solution so prepared, containing the practically pure acid, contrary to that of the crude material, does not gelatinize. The addition of small quantities of alkali causes gelatinization. For isolation the acid was first converted into the brucine salt.

BRUCINE SALT OF GUANYLIC ACID.

For this purpose the acidity of the solution was determined by titration and an equivalent amount of commercial brucine dissolved in a little alcohol was added. Crystallization began at once. Under the microscope the salt appeared as rosettes of thin colorless rectangular plates. On longer standing in the ice box more of the salt crystallized in long thin plates which were visible to the naked eye. The salt was filtered and dried.

It is very difficultly soluble in cold water, alcohol and other neutral organic solvents. It is more soluble in hot water and hot dilute alcohol. Thirty per cent alcohol is best for recrystallization. It crystallizes completely from this only on long standing. So prepared it is pure for analysis. For analysis it was dried in vacuum over phosphorus pentoxide at 110°.

0.2852 gram substance gave 29.6 cc. N at 31°, 756 mm.

0.3102 gram substance gave 0.0296 gram $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5O_5P \cdot 2C_{22}H_{34}N_2O_4$	Found.
N	10.94	11.00
P	2.69	2.65

BARIUM SALT OF GUANYLIC ACID.

For further characterization the barium salt was prepared. For this purpose the ammonium chloroform method described in the article on the thymus nucleic acid was employed. The aqueous solution of the ammonium salt was then converted by distillation with barium hydrate into the barium salt. The barium salt remained as a difficultly soluble residue. When the barium was over added a basic salt was formed which analysis showed to have approximately two molecules of barium.

The second barium is probably contained in the guanine nucleus since this is known to form a barium salt. This basic salt is more soluble in water than the neutral salt and reacts strongly alkaline. The difficulty of obtaining this pure caused us to prepare the neutral salt. For this purpose the above mixture was freed quantitatively from barium with sulphuric acid and the clear filtrate obtained treated with pure barium hydrate solution until just neutral to phenolphthalein. The neutral barium salt settled at once as a difficultly soluble white amorphous powder. It was filtered, washed rapidly with water, then successively with alcohol and ether and dried.

For the analysis it was dried *in vacuo* over phosphorus pentoxide at 110°.

0.2218 gram substance gave 0.1944 gram CO_2 ; 0.0559 gram H_2O .

0.2286 gram substance gave 0.0321 gram N (Kjeldahl).

0.2367 gram substance gave 0.0333 gram N (Kjeldahl).

0.2257 gram substance gave 0.0492 gram $Mg_2P_2O_7$.

0.2119 gram substance gave 0.0935 gram ash ($Ba_2P_2O_7$).

	Calculated for $C_{10}H_{12}N_5O_5PBa$.	Found.	
C	24.10	23.91	
H	2.41	2.80	
		I	II
N	14.06	14.06	14.05
P	6.22	6.07	
$Ba_2P_2O_7$	44.98	44.12	

When exposed to the air it absorbs carbon dioxide.

For the optical determination of the pure guanylic acid the barium salt was dissolved in acid. 0.3075 gram dry substance was dissolved in 4 cc. of *N* HCl. Total weight of the solution, 4.347 grams. In a 2 dm. tube with D-light at 25° it rotated 0.13° to the left. Calculating for the free acid and without regard for the specific gravity,

$$[\alpha]_D^{25} = -1.27^\circ$$

ON THE PREPARATION OF GLUCOSIDES.*

By W. A. JACOBS.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

For the preparation of the glucosides of sugars two general methods have been devised by Fischer.¹ In one, the alcoholic solution or suspension of the sugar is saturated with hydrochloric acid. From the resulting mixture the hydrochloric acid is neutralized with barium carbonate and removed as barium chloride. But the great expenditure of time and labor required by the repeated concentrations and extractions with alcohol led Fischer to devise a second method.² In this the sugar and alcohol are heated with a small amount of dry hydrochloric acid for thirty to fifty hours and the small amount of hydrochloric acid removed by silver oxide. In this method the reaction is not complete and moreover requires several days.

In the work which required the preparation of large quantities of these compounds, we were led to a simple modification of Fischer's first method which enabled us to prepare the glucosides within a day and in good yield. This device was used in one instance by Fischer himself,³ but was not recommended as a general method. As it may be found of service to other workers the method is described here in detail:

The solution or suspension of one part of the powdered sugar in ten parts of the dry alcohol is saturated with hydrochloric acid with cooling as in Fischer's first method. After standing one hour all reducing power disappears. The mixture is then concentrated to one-fourth its volume *in vacuo* at 20° and then poured into ordinary alcohol containing a few cubic centimeters of acetic acid. The excess of hydrochloric acid is removed by adding a fine suspension

* Received for publication, July 19, 1912.

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxvi, p. 2400.

² *Ibid.*, xxviii, p. 1145.

³ *Ibid.*, xxvii, p. 2484.

of pure lead carbonate in a little water until the mixture no longer reacts acid to congo paper. The filtrate, after treatment with hydrogen sulphide, is then concentrated *in vacuo* and the glucosides isolated as usual.

A NOTE ON THE REMOVAL OF PHOSPHOTUNGSTIC ACID FROM AQUEOUS SOLUTIONS.*

By W. A. JACOBS.

*(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)*

For the removal of the excess of phosphotungstic acid from the mother liquor, after precipitation of phosphotungstates from aqueous solutions, the usual procedure is precipitation with barium hydrate. The precipitate formed is generally very voluminous and where the mother liquor contains valuable material the loss through adsorption is apt to be great. Winterstein has already pointed out that this difficulty may be overcome by shaking out the solution with ether. In this method a mechanical difficulty enters since the solution separates into three layers, the lowest being an oily layer of ether dissolved in phosphotungstic acid, a middle aqueous layer and a top ethereal layer. The lowest layer disappears after several shakings but the operation must still be repeated many times and complete removal of the phosphotungstic acid is not assured. In amyl alcohol was found a means of quickly attaining this end. The partition coefficient of phosphotungstic acid between water and amyl alcohol is enormously in favor of the latter so that if sufficient amyl alcohol is employed after allowing time for complete separation of the layers practically all the phosphotungstic acid is removed by one shaking. If the alcohol is added in small portions the amyl alcohol phosphotungstic mixture settles as an oil. When this is repeated with fresh amyl alcohol once or twice, depending upon the amount of phosphotungstic acid present, the amyl alcohol finally floats on the top. The phosphotungstic acid is then completely removed from the aqueous solution.

When this method is to be employed one must be assured that the substances sought in the aqueous solution are not soluble in amyl alcohol. If this is the case, the addition of ether to the amyl

* Received for publication, July 19, 1912

alcohol, even to 80 per cent., may reduce this solubility without impairing the usefulness of the alcohol as an extracting agent for phosphotungstic acid. In some cases where phosphotungstates themselves are soluble in hot or cold water we have been able to decompose them by this means with a great saving of time, labor and material.

THE INFLUENCE OF HIGH PROTEIN FEEDING ON THE GENERAL METABOLISM, ON THE INTESTINAL FLORA AND ON THE BODY TEM- PERATURE OF INFANTS.

CONTENTS.

- PART I.—GENERAL CONSIDERATIONS. BY L. EMMETT HOLT, M.D., AND P. A. LEVENE, M.D.
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PART III.—BACTERIOLOGICAL INVESTIGATION. BY MARTHA WOLLSTEIN, M.D.
PART IV.—OBSERVATIONS ON METABOLISM. BY ANGELIA M. COURTNEY, ASSISTED BY JESSIE A. MOORE.

PART I. GENERAL CONSIDERATIONS.

BY L. EMMETT HOLT, M.D., AND P. A. LEVENE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, and the Babies' Hospital, New York.)

A scrutiny of the extensive data obtained from the metabolic study of infants in a state of chronic malnutrition reveals the fact that the fundamental condition is a lack of utilization by the organism of nourishment. Despite the high calorific value of the diet, there is no increase in body weight. The cause of this imperfect utilization has not yet been established. It seemed to us that there was one possible factor, the study of which has been particularly neglected, namely: the correlation of the influence of intestinal bacterial flora and the utilization of the ingested foodstuffs. An investigation of this question was the original object of the work undertaken in this study. It was planned to alter the diet of the infants in a manner that in different periods it would contain a maximum of one of the three principal food constituents—protein, fat or carbohydrate—and a minimum of the other two. This plan was carried out in one series of observations. The infant was placed on a diet of modified milk containing 2.6 per cent. fat, 3 per cent. sugar and

2.4 per cent. protein. This was continued for about three weeks. It served to determine the normal flora for this child. The diet was then changed to a milk formula containing 0 per cent. fat, 6 per cent. sugar and 3.5 per cent. protein, and later to one with 2.9 per cent. fat, 1.5 per cent. sugar and 5.8 per cent. protein.

It was observed that under the influence of these changes in diet the bacterial intestinal flora was modified both qualitatively and quantitatively. The changes are described in detail in Dr. Wollstein's paper (Part III). The gratifying feature of the observations was the marked general improvement noted during the period of high protein intake. The general condition of the infant was better than in any of the preceding periods, and the stools acquired a normal appearance, which is so rare in conditions of chronic malnutrition and which in this patient they had not previously shown.

It was intended to repeat this study on a greater number of infants. However, an unexpected observation caused us to abandon the original plan of the work and direct our energies for the present toward the analysis of that observation. This was the abrupt development of fever and nervous symptoms during the period of high protein intake, under conditions which led to the suspicion that the food given might be the cause of these phenomena.

Finkelstein¹ was the first to revive the interest of the pediatricist and the general physician in febrile conditions that are caused not by infection nor by products of bacterial action. He observed that some of the fundamental food constituents that are essential for maintenance of life and growth of the organism may, under certain conditions, exert a definite toxic effect in so far as to cause fever and other attendant symptoms of a fairly uniform character. His views were based at first on clinical observations and were later corroborated experimentally in his laboratory by his associates, principally by L. F. Meyer.² On the basis of both experimental and clinical evidence the following conclusions were formulated:

1. The normal food of infants contains what he designated as "pyretogenic" and "apyretogenic" elements.
2. The sugars and salts belong to the first group.

¹ Finkelstein, H.: *Deutsch. med. Wchnschr.*, 1909, v, 190.

² Meyer, L. F.: *Deutsch. med. Wchnschr.*, 1909, v, 194.

3. Casein and other proteins belong to the second group and never give rise to fever.

4. The fever produced by sugar and salts is due to the direct action of these substances, and not to their influence on the bacterial flora.

5. In perfect health, neither salt nor sugar, even when given in considerable excess of the normal requirement, produces any disturbance of the body temperature.

6. It is the function of the intestinal wall and of the liver to keep in check the toxic tendencies of salt and of sugar.

Our observations apparently contradicted the conclusions of Finkelstein regarding casein, and hence it became important to make certain whether or not the fever in our patient was caused by some unknown accident or was actually due to the casein intake. The subsequent observations were planned with a view of furnishing evidence in support of or against the pyretogenic influence of casein. The bacteriological studies were continued, as it was hoped that through them also some information might be obtained that would enable us to determine whether the rise of the body temperature accompanying the high intake of casein was the result of a direct action of the protein or indirectly due to its influence on the intestinal flora.

The circumstances under which the rise of body temperature first occurred were the following: In order to secure a diet containing the maximum part of the calorific requirement in form of protein, milk had to be avoided as the solvent of the casein, as such a mixture generally gave too high a calorific value to the food. The casein was therefore dissolved with the addition of a solution of sodium hydrate; the final mixture being, however, rather on the acid than on the alkaline side. This preparation (for details on composition see Miss Courtney's paper) is referred to subsequently in this article as the "synthetic" food. It varied slightly in its percentages when prepared at different times; its average composition was, however, fat, 2 per cent.; sugar, 1.8 per cent.; protein, 6 per cent.

On the fourth day, while taking this food, the infant abruptly developed high fever, great restlessness and other symptoms of discomfort, which ceased as soon as the food was changed.

Careful and repeated physical examinations of the infant were made, but failed to reveal any pathologic condition of the internal

organs to which the fever could be attributed. Hence, attention was again directed to the composition of the food during the "synthetic" period and during that of the high protein intake which immediately preceded it. The only important difference appeared to be a reduction in the amount of whey added. For six days this child had taken a milk formula containing 5.5 per cent. protein without showing the slightest disturbance. On the contrary, he was happy, comfortable and gaining in weight; but on the fourth day, on the "synthetic" diet, fever and its attendant symptoms were seen.

The same observation was repeated on this child two months later, with results which were practically identical. After the temperature had been normal for a long time and the child to all appearance well, he developed, on the sixth day after beginning the "synthetic" food, high fever, accompanied by the symptoms previously seen. All these, as before, disappeared immediately with the change in food.

Observations on another infant gave essentially the same result. In this patient the protein percentage in the milk formula was gradually raised in the course of thirteen days from 4 to 6 per cent. It was kept at the latter figure for six days. During all of this period of high protein feeding, lasting nineteen days, the child remained well and gained weight. The "synthetic" food formula was then substituted, and promptly on the fourth day thereafter the child responded with an attack of fever. Since the symptoms were not so severe in this patient, the food was not changed as soon as fever occurred. As rather marked constipation existed, castor oil and then calomel were administered to determine whether or not constipation might be a factor. But the temperature was not influenced by the catharsis. Finally, on the seventh day of the fever, the food was discontinued and a simple milk dilution substituted. Immediately the temperature fell to normal and remained there.

On a third infant two observations were made. In the first one the protein percentage in the milk formula was gradually raised from 4 to 6 per cent. during a period of ten days. It was kept at the latter figure for five days longer. During this time there was no fever or sign of disturbance and a slight gain in weight. On the

fifth day, after a change was made to the "synthetic" food, fever and the usual symptoms developed, ceasing at once when the food was changed to a simple milk dilution.

In a second observation on this infant a milk formula containing 6 per cent. protein was continued for sixteen days without fever or abnormal symptoms; but fever developed on the fifth day after a change was made to the "synthetic" food, which contained the same percentage of protein.

From the standpoint of metabolism, the only striking thing accompanying the fever was the complete retention of the chlorids. This was seen in all the cases. It began two or three days before the rise in temperature occurred and continued into the febrile period. The nitrogen retention was good, and the proportion of ethereal sulphates in the urine did not differ strikingly from the values obtained in other periods.

ANALYSIS OF THE FACTORS THAT MIGHT HAVE BEEN INSTRUMENTAL IN CAUSING THE FEVER.

That its occurrence was not accidental seems established by its regular development in every observation at approximately the same time after beginning the "synthetic" food; also by the fact that although these patients were under observation for several months, no similar attacks were seen under other conditions; nor was there evidence in any case that the fever was due to intercurrent illness as a complication. This possibility was always borne in mind; the patients were frequently examined and watched for local symptoms of every sort. As already mentioned, in only one case was constipation marked, and in this patient free catharsis did not influence the fever. Furthermore, the stools in most of the observations, both before and during the fever, were quite normal.

The changes in the intestinal flora were very similar in all the observations. There was a very great reduction in the number of fermentative organisms (it will be remembered that in all the cases the percentage of carbohydrates in the food was low, usually 2 per cent. or less), and a great increase in the proteolytic varieties. That the fever was due to infection from the latter seems highly im-

probable for several reasons: The flora during the attack did not differ essentially from that which existed for several days before and several days after the fever; the symptoms developed very acutely, and if the food was at once changed they ceased almost immediately; furthermore, the stools gave no evidence of any such infective process.

The rise of temperature invariably occurred after the "synthetic" food had been given and on no other diet, and that it came regularly in each case after the food had been taken for about the same length of time points strongly to the food as the cause of the temperature. What was it in the food, then, which brought about this result? Was it simply cumulative effect of previous high protein feeding, or was it something in the "synthetic" food mixture?

The first hypothesis seems disproved by two of the observations. Whereas, in the others the temperature occurred in about ten days after beginning the high protein feeding, in two instances very high protein (in one child, between 5 and 6 per cent. for eleven days, in the other 6 per cent. for sixteen days) had been given without producing fever or other abnormal symptoms. Yet in both fever developed on the fourth day after the change was made to the "synthetic" formula.

The "synthetic" food formula did not differ essentially in the percentage of fat, sugar and protein from the food which immediately preceded it and which produced no fever. On the contrary it was the aim to make the percentages in the two foods as nearly identical as possible. The only difference was in the mode of preparation. To the "synthetic" food to effect a solution of the casein in the minimum amount of whey, sodium hydrate was added. The daily amount used was about 1 gm. In spite of this addition the total salt intake of the infant while on the "synthetic" food differed but little from that taken in the period immediately preceding. Thus the average salt intake of Case 1 for ten days before the "synthetic" food was 12.2 gm.; during the four days of the "synthetic" period it was 10.9 gm. In Case 2 the intake in the antecedent period averaged 6.9 gm.; during the "synthetic" period it averaged 7.1 gm. In Case 3 the average intake for eighteen days

before the "synthetic" period was 9.03 gm.; for the five days of the "synthetic" period it averaged 9.50 gm.

It would appear, therefore, that the total salt intake could not be regarded as a factor in producing the fever. That the sodium hydrate added was of itself sufficient to cause a rise in temperature seemed most improbable. One observation was made, however, which bears on this point. Since sodium hydroxid was used to neutralize the phosphoric acid of the casein, sodium phosphate was added to a diet which had caused no fever (fat, 2 per cent.; sugar, 6.5 per cent.; protein, 2.6 per cent.); this was continued for four days without result so far as fever was concerned.

Inasmuch as the "synthetic" food was low in sugar (about 2 per cent.), although not lower than the food used in the period immediately preceding it, one observation was made on Case 3 during the febrile period to determine whether the addition of lactose to make the proportion in the food 5 per cent. would affect the fever. This was continued for two days without result. But two days later the fever ceased, twenty-four hours after the food had been changed.

We come now to a consideration of the amount of whey in the "synthetic" food formula as compared with that used in the other food formulas containing high protein. The latter were prepared (*vide* Miss Courtney's paper, Part IV) after the manner of Finkelstein's *Eiweissmilch*. In 1,500 c.c. of this food which were prepared for one day, there were used 600 c.c. of milk; while in the "synthetic" formula but 200 c.c. of milk. It will be remembered that no child took over 1,260 c.c. daily, and therefore the whey received was less than 175 c.c. It seems to us that the most probable explanation of the fever observed in these infants is the administration of the large amounts of protein without a sufficient amount of whey.

As to the actual significance of the retention of the chlorids, we have at present no definite information. It is, however, noteworthy that the chlorid intake in the "synthetic" food averaged about 0.50 gm., while that on the other foods was nearly three times as great; but such a reduction of the chlorids in normal infants did not bring about a complete disappearance of the chlorids from the urine.

CONCLUSIONS.

1. Alimentary fever may under certain conditions occur in infants after administration of casein of cow's milk and perhaps of other proteins.

2. The rise of the body temperature was observed only when the food mixture was made up so as to contain about 6 per cent. of protein (chiefly casein) and a minimal quantity, only about 150 to 175 c.c., of milk daily.

3. The rise of body temperature was invariably accompanied by a retention of chlorids, which, however, usually preceded the febrile attack by two or three days.

4. After the first rise of temperature the fever persisted so long as the diet was continued, but in every instance promptly disappeared as soon as the food was changed.

5. The fever is apparently due to the direct action of the absorbed protein, since the bacterial conditions of the intestines noted during the period of the "synthetic" food were not different from those noted during the preceding period. Also, Conclusion 4 speaks in support of this view.

6. From the observations here reported it would appear that the changes in the food influenced the chemistry of the excreta more than they did the bacteriology. For while the chemical changes resulting from the food variations differed considerably in the individual cases, the bacteriology changed along the same general lines in all. In view of this fact and of the slow changes in the intestinal flora noted in these observations, such changes would seem to have but a limited application for therapeutic purposes, though a very definite one when the food changes can be made sufficiently great.

7. It seems clear from these observations that one may use considerably higher percentages of protein in milk formulas than the 3.5 per cent. of Finkelstein's formula. This is a point which may be of much practical importance in conditions in which there is marked intolerance both of fats and carbohydrates. Such high proteins as 4.5 per cent. or over should only be used for limited periods, and never given at all except with a suitable proportion of whey.

8. This report emphasizes the physiologic importance of whey in

the nutrition of infants and other young animals. While laying stress on the dangers of whey, especially from its sugar content, in many forms of intestinal disturbance, Finkelstein and Meyer have also appreciated the dangers to nutrition which may follow reduction of the salts. Recently Osborne and Mendel⁸ also have found that the mineral salts in the proportion present in whey were absolutely essential for the growth of animals, and that in the absence of salts, though maintenance of life was possible, growth could not be induced. As yet it is not possible to formulate any definite opinion as to the nature of the whey components which inhibit that action of casein, which, in the absence of whey, causes the rise of body temperature.

9. The present observations call special attention to the work of V. C. Vaughan⁴ and his collaborators, who have repeatedly reported the production of fever by the subcutaneous injection of various proteins. Whether or not the fever induced clinically in our cases and experimentally by Vaughan is occasioned by the same mechanism, remains to be established.

PART II. CLINICAL OBSERVATIONS.

By ALAN BROWN, M.D.

(Resident Physician to the Babies' Hospital, New York.)

CASE 1.—Francis H. was admitted as a case of marasmus July 11, 1911, aged 8 months, having suffered from digestive disturbances, chiefly intestinal, since 3 months of age. At the time of admission he weighed but 3,850 gm., had no acute symptoms, but was suffering from chronic otitis and was very backward in physical development. During the next two months his digestion and general condition slowly improved. There was an initial loss in weight of 350 gm. till July 24, when he touched his lowest point, 3,500 gm.; from this time there was a steady but slow increase in weight amounting to over 800 gm., and he was taking a formula made from whole milk, containing fat, 2.6 per cent.; sugar, 5 per cent.; protein, 2.4 per cent.; 180 c.c. for each of seven feedings. His stools for the most part were good; no acute symptoms were present.

⁸Osborne, T. B., and Mendel, L. B.: Carnegie Inst. of Washington, Pub. 156, 1911, i and ii.

⁴Vaughan, V. C., Cumming, J. G., and Wright, J. H.: Ztschr. f. Immunitätsforsch., 1911, ix, 458; Vaughan, Cumming, and McGlumphy, C. B.: *ibid.*, 1911, ix, 16.

Metabolism observations were begun on September 14, at which time his weight was 4,330 gm. His appetite was excellent, there was no vomiting and his stools were 3 to 4 daily, usually yellow in color and seldom contained mucus. No change in food was made for two weeks. He continued to gain in weight, his stools were for the most part yellow and smooth, but at times became thin and loose. The number greatly increased while in the metabolism bed; there were eight to eleven daily, but most of them were small. The temperature remained normal and the child was comfortable and happy.

On October 1 the food was changed to a formula consisting of fat, 2.6 per cent.; sugar, 3.0 per cent.; protein, 2.4 per cent. The reduction in sugar was made on account of the rather frequent, thin stools. This food was continued for three weeks. The stools were less frequent but still somewhat thinner than normal and yellow, averaging from seven to nine daily. The child was kept in the metabolism bed, except for an interval of two days, the weight up to this time being about stationary.

October 14 the food was changed to fat, 0 per cent.; sugar, 6 per cent.; protein, 3.5 per cent. This was fat-free cow's milk with the addition of 1.5 per cent. cane sugar. This food was continued for five days, during which time he gained 250 gm. in weight and seemed perfectly comfortable. The stools showed, however, a marked and immediate change; they became large, loose and contained quantities of greenish-brown jelly-like mucus; there were from eleven to thirteen daily.

October 19 the food was made fat, 1.6 per cent.; sugar, 4.7 per cent.; protein, 3 per cent. This formula was obtained from partially skimmed milk with the addition of .75 per cent. of cane sugar. This was continued for five days. With this change the amount of mucus somewhat diminished, the stools were reduced to eight a day and they were not so thin. The child lost during the five days about 100 gm. in weight.

October 25 he was given casein milk (*Eiweissmilch*), three parts, and barley-water, one part, the formula being fat, 1.9 per cent.; sugar, 2.3 per cent.; protein, 2.9 per cent. The effect of the reduction in the sugar was immediate and very striking. The stools became smooth, soft, brown, formed, from three to four a day, and the amount of water in the stools which had been from 100 to 300 c.c. daily was reduced to 36 to 43 c.c. a day.

October 31 his food was changed to a formula consisting of fat, 3.6 per cent.; sugar, 3.1 per cent.; protein, 5.6 per cent. This was continued for one week; during this time the stools were gray, yellow, dry and formed, from four to five daily. He more than regained his lost weight, having reached 4,980 gm. His first metabolism period closed November 6.

During the interval between November 6 and 16 he was out of the metabolism bed and was being fed casein milk of the following percentages: fat, 2.75; sugar, 1.8; protein, 3.9. The weight remained stationary and the stools averaged three to four a day, being yellow, pasty and at times formed.

November 16 he was again put in the metabolism bed and the food was changed to the following: fat, 2.9 per cent.; sugar, 1.5 per cent.; protein, 5.8 per cent.; this is referred to in the chemical report as the "synthetic" food. Up to this time the general condition of the patient was excellent; he was happy, comfortable and did not appear to be disturbed by the restraint of the metabolism

bed. For the first four days he took his food well. The character of the stools, on this new food, changed considerably. They became gray, formed, dry and crumbly, occasionally being coated with mucus. The frequency remained about the same, three to four daily. On the nineteenth he refused 120 c.c. of the 1,260 c.c. offered, but showed no other signs of disturbance. On the morning of the twentieth the temperature was 100.2° F.; he vomited once and took less than half of his usual food. He was restless, fretful, irritable, and had a slight cough and a few coarse râles in the chest. His slight bronchitis was thought sufficient to explain his symptoms. The course of his temperature is shown on the accompanying chart (Chart 1). In the early morning of the twenty-first it was 105.2° F.; the child was pale, considerably prostrated, breathing rapidly, restless and continually rolling his head from side to side. His symptoms suggested the beginning of a pneumonia, although the only signs in the chest were a few râles. The suspicion was strengthened on examination of the blood which showed a leukocytosis of 27,160. Further evidence was shown by examination of the urine which showed a total absence of chlorids.

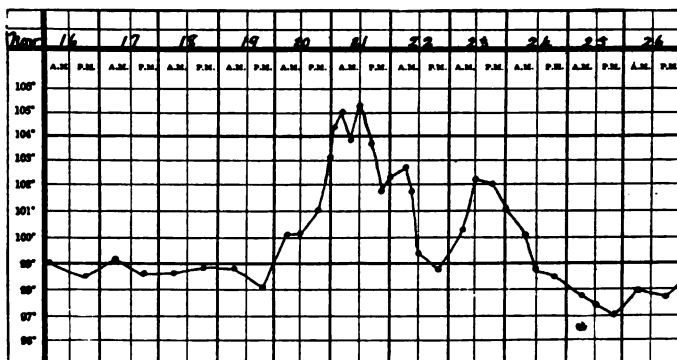


CHART I. Temperature of F. H.; first attack of fever.

He was immediately removed from the metabolism bed, given castor oil, the colon was irrigated and the child put on a food consisting of 1 part of whole milk and 3 parts water, with lactose added; formula: fat, 1 per cent.; sugar, 5 per cent.; protein, .9 per cent. Under this régime he rapidly improved and in two days was apparently as well as ever. During this period, November 16 to 21, he lost in weight 447 gm. The next blood examination made November 25 showed the leukocytes to be 9,200.

Between November 23 and December 11 he was fed on dilutions of whole milk, till on December 8 he was taking a food consisting of fat, 3 per cent.; sugar, 4.5 per cent.; protein, 2.6, with the addition of 24 c.c. of maltose and 6 c.c. of olive oil daily. His stools averaged two to three a day, were generally yellow and smooth.

He was again put up in the metabolism bed on December 12, the food being fat, 2 per cent.; sugar, 6.4 per cent.; protein, 2.5 per cent. Of the sugar, 1.25 per cent. was maltose, also mono- and di-sodium phosphate were added. This

was continued for four days without any effect on the temperature or on his other symptoms so far as could be determined. He was removed from metabolism bed December 23, having gained about 120 gm. during the period.

December 26, after an interval of three days he was again put in the metabolism bed and given the "synthetic" food containing fat, 2.1 per cent.; sugar, 1.7 per cent.; protein, 6.3 per cent., 1,260 c.c. daily. A blood-examination made two days later showed a leukocytosis of 24,400, but this was possibly explained, in part at least, by a slight otitis. After the first day he took his new food well and his weight remained nearly constant. The stools were from four to five a day, light gray and formed, with occasionally a slight amount of mucus. He was comfortable, quiet and exhibited no nervous symptoms.

December 31 he refused nearly all of his food and he seemed somewhat out of sorts; in the evening a sharp rise of temperature occurred to 103.8° F. and a repetition of practically all of the symptoms noticed in his previous disturbance on a similar food November 21.



CHART 2. Temperature of F. H.; second attack of fever.

A dose of castor oil, and an immediate change of food to diluted whole milk were followed by a prompt fall in temperature and a speedy disappearance of all his symptoms. A temporary drop in weight of nearly 700 gm. occurred during this upset, but this was quickly regained and for the next five days his appetite was good, his stools normal, and his general condition excellent.

On January 25 he was discharged from the hospital in good condition. His progress after the last observation was without event and no disturbance of digestion occurred. His daily weights during the period of observation are given in Chart 3.

CASE 2.—Jacob S. was admitted Dec. 11, 1911, aged 5 months; suffering from malnutrition; weight 3,437 gm. The patient had gained little since birth, his digestive symptoms being chronic constipation and frequent vomiting. For the first two and one-half months in the hospital he was fed chiefly on dilutions of whole milk, with small amounts of maltose added. His digestive symptoms steadily improved. His stools averaged two to three a day, usually smooth and pasty, and he gained 551 gm. in weight.

February 27 he was put up in the metabolism bed and given a milk mixture whose formula was fat, 1.45 per cent.; sugar, 5 per cent.; protein, 3.5 per cent. He was given 150 c.c. for each of seven feedings, with 15 c.c. of olive oil every day. Two days later the protein percentage was increased and the sugar decreased, the formula being: fat, 1.45 per cent.; sugar, 4.5 per cent.; protein, 4.0 per cent. The percentage of protein was gradually raised and that of the sugar lowered at three day intervals until on March 13 he was taking fat, 1.45 per cent.; sugar, 2.1 per cent.; protein, 6 per cent., made after the manner of casein milk. On March 11 the olive oil was reduced to 6 c.c. and 5 per cent. lime water was added on account of occasional vomiting.

March 18 the food was changed to the "synthetic" food: fat, 1.45 per cent.; sugar, 1.8 per cent.; protein, 6 per cent. Up to this point he had done extremely well; had gained 501 gm. in weight and averaged three to four stools daily, which were for the most part yellow, pasty, but small, and inclined to constipation. He

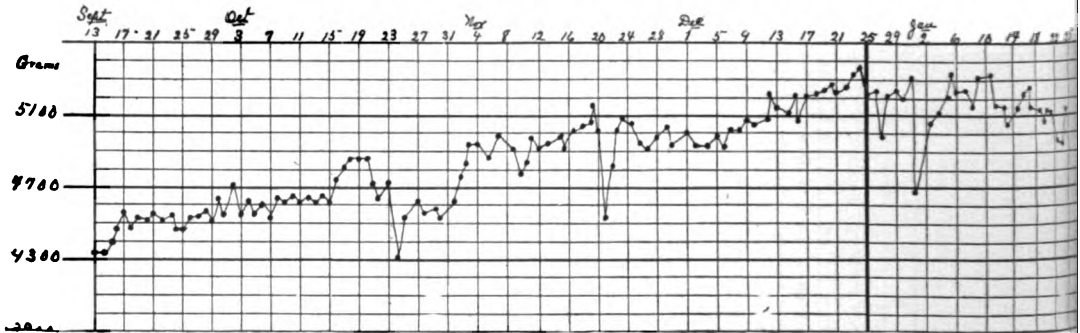


CHART 3. Daily weight chart of F. H.

manifested no unusual symptoms and appeared in every way normal. After taking this food well for three days he showed his first sign of disturbance, which was to refuse some of his food. At noon of the following day (twenty-first) the temperature rose to 99.4° F.; at 7 p. m. it was 103.2° F.; it fluctuated irregularly for the next week, usually being between 100° and 102° F. During this period (up to March 27) his food was unchanged but he took it badly, frequently leaving more than one-half of the amount offered. The stools averaged three to six a day; they were small, of a yellow-brown color, often dry, hard balls, quite different from those of Case 1 on the same food.

It was thought that his constipation might be the explanation of his temperature. He was given two drachms of castor oil, and later calomel. The stools following catharsis were loose and contained considerable mucus, but the temperature continued. Careful and repeated physical examinations revealed no local cause for his temperature. He had few symptoms except a moderate amount of irritability, marked anorexia and a rapid loss in weight.

Beginning March 25, 30 c.c. breast milk were given before each feeding. This produced no change in the symptoms and finally on March 27 the "synthetic" food was stopped; whereupon the temperature rapidly fell to normal and re-

mained so, except for one slight rise several days later, due to antitoxin. He rapidly regained his appetite and in three or four days was gaining steadily in weight.

The blood-examinations made are given in a subsequent table.

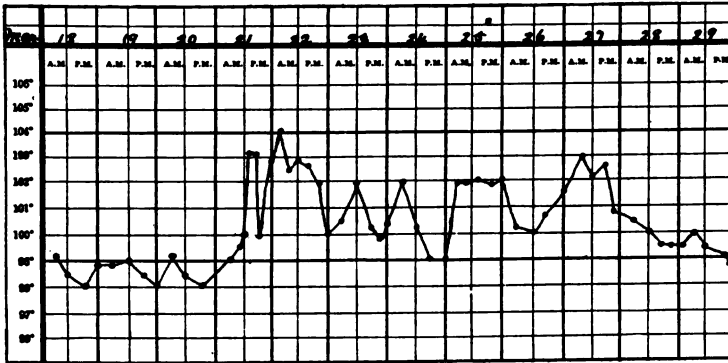


CHART 4. Temperature of J. S.

The patient was discharged from the hospital six weeks later in good general condition, weighing 4,471 gm.

For daily weight record during the period of observation see Chart 5.

CASE 3.—F. D. was admitted September 14, 1911, aged 4 months, suffering from chronic indigestion, weight 3,045 gm. From this date, except for one slight upset in December, his digestion and general condition improved; he took his

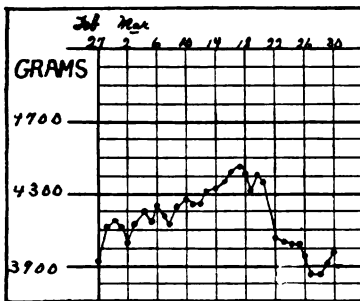


Chart 5.

CHART 5. Daily weight chart of J. S.

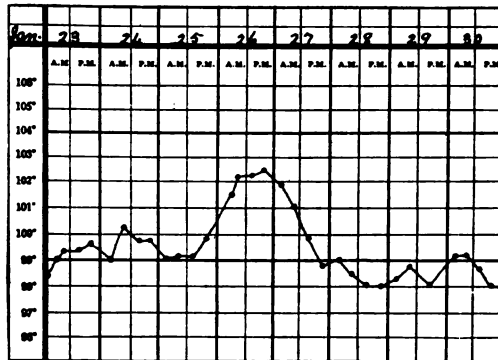


Chart 6.

CHART 6. Temperature of F. D.; first attack of fever.

food well, vomited but seldom, and his stools averaged three to four a day, were brown, pasty, at times somewhat constipated. His feedings consisted for the most part of dilutions of whole milk; his best weight, in December, showed a gain of 1,230 gm.

January 5 he was put in the metabolism bed and given a food consisting of fat, 1.5 per cent.; sugar, 2.2 per cent.; protein, 3.4 per cent. The protein was gradually increased at three day intervals to 6 per cent. on January 18. He gained 164 gm., was happy and comfortable and his stools averaged six to seven a day, usually gray and pasty, rarely loose.

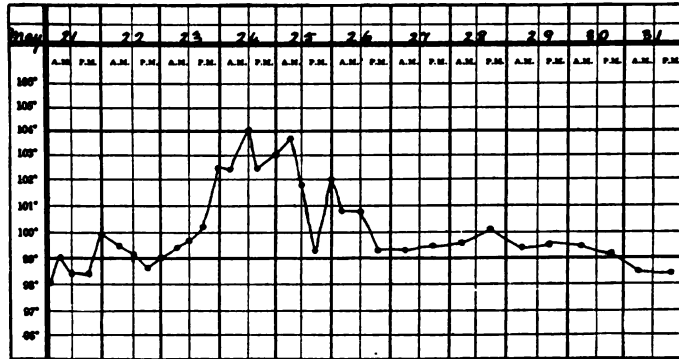


CHART 7. Temperature of F. D.; second attack of fever.

January 22 he was given the "synthetic" food; fat, 1.8 per cent.; sugar, 1.8 per cent.; protein, 5.9 per cent. On the evening of January 25 his temperature rose to 99.8° F. and in the morning of the following day to 101.6° F. The fever was accompanied by irritability, restlessness, rapid breathing, and considerable prostration. He was immediately removed from the metabolism bed, given a cathartic and the food changed to a formula made from whole milk containing

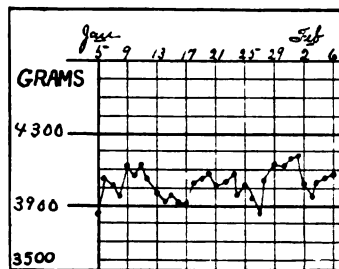


Chart 8.

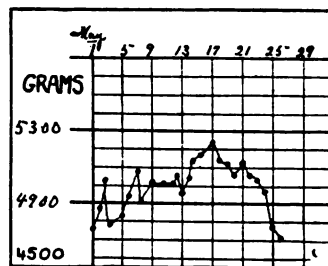


Chart 9.

CHARTS 8 AND 9. Daily weight charts of F. D.

of fat, 1.5 per cent.; sugar, 5 per cent.; protein, 1.4 per cent. These measures were followed by an almost immediate change for the better. The loss in weight of 180 gm. was quickly regained and he continued to improve till his discharge on March 5, when his general condition was excellent.

March 19 the patient was readmitted with symptoms similar to those with which he first entered the hospital. For the following six weeks his digestion

and general condition improved and he gained 720 gm. For the most part his food consisted of various dilutions of whole milk with sugar added and his stools were good.

On May 1 he was put in the metabolism bed, his food being: 2.6 per cent. fat, 4.5 per cent. sugar, 5 per cent. protein, and in addition was given 7.5 c.c. of olive oil each day. The protein in his milk was increased to 6 per cent. on May 4 and then kept at this point. During the interval between May 1 and 20 he gained 350 gm. His stools averaged two to three a day, mostly yellow and pasty, at times dry and constipated, requiring an occasional dose of magnesia. In his general condition he improved steadily.

On May 20 he was put on the "synthetic" food; except for the existence of constipation nothing noteworthy happened until the evening of May 23 when the temperature rose to 101.8° F., but no other manifestations occurred till the afternoon of May 24, when he was noticed to be restless, refused part of his food and vomited once. On May 24 lactose to 5 per cent. was added to the "synthetic" formula but with no apparent effect on the symptoms. On the morning of May 25 the symptoms practically identical with those observed on previous similar occasions with other children developed but of greater severity. He was immediately removed from the metabolism bed, given a cathartic and the food changed to a dilution of whole milk. With these measures all the symptoms speedily disappeared and in thirty-six hours he was apparently as well as ever.

He was discharged June 27 in good general condition, having gained 1,740 gm. since March 20.

In Table I are given the results of the various blood-examinations made in the different cases.

Case 1 in the first period with high temperature showed marked polymorphonuclear leukocytosis,⁵ which quickly disappeared when the temperature became normal. In the second period the variations during the fever from the average for the child were so slight as to be without significance.

Case 2 showed no marked variations during the febrile period from the findings before and subsequent to it.

Case 3 showed a distinct leukocytosis coming with the fever and disappearing with it. In the second observation on this patient the same thing was seen, the differential count being inconclusive.

SUMMARY.

In every one of the five observations made on the three infants, an attack of fever followed the administration of the "synthetic" food, although in all the temperature had been previously normal

⁵ It is to be borne in mind that in infants of this age and type the lymphocytes normally exceed in number the polymorphonuclear cells.

TABLE 1.
Results of Blood-Examinations in the Three Cases.

Case.	Period.	Condition.	Total Number Leukocytes.	Polymor- pho- Nuclear, per cent.	Lymphocytes, per cent.	Remarks.
F. H.	Nov. 21.	First day of fever	27,160	57.6	37.0
	Nov. 25.	First day of normal T.	9,240	37.3	54.0
	Dec. 30.	Normal temperature	20,200	46.0	49.0	Slight otitis present
	Dec. 31.	Fever beginning	17,900	42.0	52.0
	Jan. 1.	Fever high	17,400	49.5	41.5
	Jan. 2.	No fever	18,200	46.0	45.3
	Jan. 3.	No fever	14,200	39.2	49.5
	Jan. 4.	No fever	13,800	43.0	47.0
	Jan. 5-8.	No fever	16,400	47.3	46.9	Average for four days
J. S.	March 13.	No fever	18,400	42.0	51.5
	March 16.	No fever	18,800	51.0	49.0
	March 20.	No fever	19,800
	March 21.	Fever began
	March 23.	Fever present	14,400	57.0	43.0
	March 24.	Fever present	16,400	42.0	57.0
F. D.	Jan. 22-25.	No fever	8,700	Average for four days
	Jan. 26.	Fever began	10,700
	Jan. 27.	Fever present	14,300
	Jan. 28.	No fever	10,000
	Jan. 29.	No fever	8,300
	May 21-23.	No fever	15,600	54.3	42.0	Average for three days
	May 24.	Fever high	20,000	50.0	50.0
	May 25.	Fever high	20,000	46.0	54.0
	May 26.	Fever slight	18,200	44.6	55.0
	May 27.	No fever	12,800	42.0	58.0
	May 29.	No fever	9,200	47.3	52.6

for a considerable period. Three times this developed on the fourth day, once on the fifth and once on the sixth day. In four observations the patients had previously taken as high, or nearly as high, a percentage of protein in food prepared differently for much longer periods (in one case for sixteen days) without the development of fever. In every instance the fever developed abruptly without other warning than anorexia and some restlessness which usually preceded the rise of temperature for about twelve hours. The fever persisted so long as the diet was continued, but ceased almost at once when the food was changed to a simple milk dilution. With the fall in temperature all of the general symptoms speedily disappeared.

The highest temperature noted was 105.4° F. In the patient

showing the least reaction it rose to 102.5° F. The average rise was to 104° F. Marked nervous symptoms usually accompanied the fever; constant rolling of the head, extreme restlessness, pallor, rapid respiration, considerable prostration, and in three observations marked leukocytosis. In one case the white cell count reached 27,000. Digestive symptoms, except anorexia, were conspicuously absent. Initial vomiting was noted but once; the bowels were usually somewhat constipated but not otherwise abnormal; a moderate abdominal distention was noted but once; neither pain nor tenderness was evident in any case. The urine showed several times an increased indican reaction, but this was not constant nor persistent. Pallor, rapid respiration and prostration were marked in all but one case. In every instance there was rapid loss of weight, which was due partly to the refusal of food and partly to catharsis. This loss was soon repaired in nearly every case. In none of the observations did the symptoms resemble an ordinary attack of acute indigestion.

PART III. BACTERIOLOGIC OBSERVATIONS.

By MARTHA WOLLSTEIN, M.D.

(*Pathologist to the Babies' Hospital, New York.*)

Throughout these five periods of observation the method of procedure was the same. Specimens of feces obtained from the rectum were studied bacteriologically for several days before the chemical examination of the excreta was begun. This served the purpose of establishing a control for each child. Throughout each period of study daily examinations of the feces were made.

TECHNIC.

The samples of feces were obtained in the usual way, by introducing into the rectum a glass tube of narrow caliber, plugged at one end and sterilized. As a rule, the tube became filled with fecal matter within a few seconds, but during the periods of constipation it was difficult to obtain more than a small amount, sometimes less than half a cubic centimeter, in several minutes. Occasionally no

material at all could be obtained even in more than one attempt. From the fecal specimens smears were prepared and stained by Gram's method, the remainder of the material being at once emulsified in neutral, dextrose-free broth. The emulsion was made as thorough as possible, and care was taken lest it be too thick. This emulsion served to inoculate glucose and lactose fermentation tubes, whole milk tubes and tubes of the acid-dextrose-broth introduced by Heymann, and described by Finkelstein⁶ as consisting of 2 per cent. dextrose in 0.5 to 1 per cent. acetic acid broth. Agar and gelatin plates were also poured from the tube of emulsified feces, which was then itself incubated and compared with the growths in the acid broth on the following day. The cultures were incubated both aerobically and anaerobically, but it was soon found that anaerobic cultivation of plates poured directly from the fecal emulsion was utterly useless, since the colon varieties overgrew all other forms. Anaerobic incubation was, therefore, reserved for the cultivation of second or later generations from the acid broth or fermentation tubes, and of colonies isolated from deep agar sticks and grown further on plates or in fluid media.

It may be said at the outset that the varieties of bacteria isolated from the stools of these three infants were not numerous. There was a marked sameness noted in the bacteriology throughout all the observations, a fact which was to be expected since the food was practically the same in the parallel stages of the five observations. While by no means every variety of bacterium seen in smears and cultures from the feces was isolated in pure growth, the great majority were certainly so obtained. The most useful media proved to be the lactose broth in the fermentation tubes, and the acid-dextrose broth or Heymann's medium. Gelatin plates were useful in giving a general idea of the varieties of liquefying organisms present. Whole milk tubes proved to be disappointing as indicators of the presence of proteolytic bacilli, for the reason that streptococci which peptonized milk were very numerous in the feces of these children, and it was repeatedly noted that on a diet rich in carbohydrates, where the cultures showed the presence of a fermentative and not a proteolytic flora, the amount of gas in the fermentation

⁶ Finkelstein: Deutsch. med. Wchnschr., 1900, xxvi, 263.

tubes was comparatively low as was to be expected, but the milk tubes were more than 80 per cent. peptonized. As a control a breast-fed baby was studied. This child showed the typical bifidus picture in smears and cultures from the stool. Only 0.5 to 1 c.c. of gas was formed in the lactose fermentation tube, and yet the milk tubes were almost completely peptonized. The liquefying streptococcus was found in large numbers in the stool from this infant.

BACTERIOLOGY OF THE FOOD.

In order to determine whether the bacteriologic intake with the food bore any relationship to the fecal flora, samples of the food were studied at intervals. At the beginning of the first observation, September 13, 1911, the prepared food contained *Streptococcus*, *Albococcus*, *B. lactis aerogenes* and a liquefying, fluorescent bacillus. There were 12,000 colonies to 1 cubic centimeter of food. Nine days later the number of colonies had risen to 3,500,000 per c.c., and *B. subtilis* was isolated as well as the other varieties. It became obvious that a cleaner milk was needed, and a change was made on October 11. The number of colonies in the new milk used numbered only 1,600 to the cubic centimeter, and were composed only of staphylococci and streptococci. The food after it had been prepared for the feeding of the child, however, contained 16,000 to 19,000 colonies to the cubic centimeter, and on several successive days *Streptococcus*, *Aurococcus*, *Albococcus* and *B. coli communior* were found. Greater cleanliness in handling of the bottles was then instituted, and after November 1 no further contaminations appeared in the food. The protein milk contained the largest numbers of bacteria, 200,000 to the cubic centimeter being present on one occasion. This was reduced to 30,000 later. From the ripened milk used in preparing the protein milk a long gram-positive bacillus was isolated. It grew very faintly on agar, fermented lactose, and coagulated milk softly without peptonizing the curd to any extent. This bacillus was looked on as *B. bulgaricus*, described as a powerful lactic acid ferment by Cohendy,⁷ and with which the milk was said to be ripened. Herter and Kendall⁸ found this bacillus in the feces of a rhesus monkey fed on *bacillac*.

⁷ Cohendy, M., Compt. rend. Soc. de biol., 1906, lx, 558.

⁸ Herter and Kendall: Jour. Biol. Chem., 1908-9, v, 293.

The "synthetic" food never contained over 2,000 colonies to the cubic centimeter.

Five observations were made on three children. The details of the bacteriologic findings during the course of the studies follow:

Observation 1.—F. H., 9½ months old. Sept. 13, 1911. Food, modified milk having percentages of fat, 2.6; sugar, 5; protein, 2.4; containing albococci, streptococci, fluorescent bacilli which liquefy gelatin, and *B. lactis aerogenes*.

Feces yellow, no mucus present. The material was granular and did not make a smooth emulsion in dextrose-free broth. Smear shows oval cocci in pairs and chains, slender, gram-positive bacilli, some with pointed, tapering ends (*acidophilus*); granular or vacuolated bacilli, some with poles staining deeply; gram-negative bacilli like *B. coli* forming a background for the larger, blue, bacillary forms. No spores were seen.

From agar plates an oval streptococcus, the *Enterococcus*, and *B. coli communis* were isolated. In the fermentation tubes small numbers of *B. bifidus* grew, and were subcultured, though not isolated, in acid broth in the anaerobic jar (pyrogallic acid—hydrogen—vacuum method). *B. acidophilus* was isolated from the acid broth tube. Only 1.5 c.c. of gas formed in the fermentation tubes of lactose broth and 1.25 c.c. in the glucose broth. Milk was coagulated and slightly peptonized.

September 16.—Three days later there were more blue than red bacteria noted in the gram-stained smear, and in the cultures *staphylococci*, *streptococci*, *B. acidophilus*, *B. bifidus*, *B. coli communis*, and *B. fluorescens* liquefying gelatin were noted. Only 1 to 1.5 c.c. of gas formed in the fermentation tubes, and whole milk was markedly peptonized, only a small solid coagulum remaining in the bottom of the tube. On gelatin plates liquefying green colonies and liquefying white colonies developed of fluorescent bacilli and albococci.

This picture varied but slightly during the following week, *B. coli communis* being found one day and a strain of the Shiga (non-mannite fermenting) type of *B. dysenteriae* another day. The history disclosed the fact that the child had suffered from "loose bowels" for a period of four weeks before admission, which was sought because of vomiting, diarrhea and colic.

September 23. *B. subtilis* was found in the feces. The colon bacilli were very numerous, but *B. bifidus* was present in comparatively smaller numbers. These relative proportions continued until the change to cleaner milk October 11, four days after which date no more *subtilis* bacilli were noted.

October 15. Food, milk with percentages, fat, 0; sugar, 6; protein, 3.50.

October 16. Cocci and colon bacilli were very numerous; *B. acidophilus* was present and *B. bifidus* had increased in numbers. *B. lactis aerogenes* was also found. The amount of gas produced was small and milk was coagulated but only very slightly peptonized.

October 19. Food changed to fat, 1.6; sugar, 4.5; protein, 3.

The numbers of *B. coli* were very numerous, *B. communior* being found on several successive days. *Staphylococci*, *diplococci* and *streptococci* were present in very large numbers.

October 20. *B. perfringens* (*B. aerogenes capsulatus*, Welch) was noted.

October 23. A fluorescent, liquefying bacillus was found on gelatin plates.

October 24. Food changed to protein milk, diluted with barley water: fat, 1.9; sugar, 2.3; protein, 2.9, but the child refused it for twenty-four hours.

October 25. A specimen was obtained after the child had fasted one whole day. The smear showed an equal number of gram-negative and gram-positive bacteria, having hitherto always shown more of the Gram-positive type. The change was due to the small numbers of cocci present, while colon-like bacilli were numerous and some gram-positive bacilli, apparently *B. aerogenes capsulatus*, were seen. The amount of gas in the fermentation tubes was more than twice as great as it had ever been, and in them *B. aerogenes capsulatus* was growing together with colon bacilli and cocci.

October 26. A long gram-positive bacillus was present in addition to the other varieties. This bacillus was apparently identical with the *B. bulgaricus* in the food, which was made up with ripened milk.

The only change noted in the next few days was a marked increase in the amount of gas found in the fermentation tubes, no new varieties of bacteria being noted.

October 31. Food changed to fat, 3.6; sugar, 3.1; protein, 5.5.

The amount of gas grew larger, the cocci decreased in numbers while colon bacilli and gas bacilli were numerous. There were very few bifidus forms seen.

November 6. Food of the same formula, but three drachms of maltose were added to relieve the constipation. From the dry maltose a large, gram-positive spore-bearing bacillus identified as *B. megatherium* was isolated. The day following the addition of the maltose to the food this spore-bearing bacillus was present in the feces. Castor-oil was given.

November 8. Cocci were very numerous; many colon bacilli and large spore-bearing, gram-positive bacilli were also seen. The spores in some of these bacilli were oval in shape, situated in the center of the organism, giving the whole bacillus an oval form; while in others a round spore lay at one end, giving the bacillus a nail-shape; in still others the spore was situated in the center of the bacillus and was narrower than the body of the rod, thus not changing its shape. Two forms of spore-bearing bacilli were isolated and identified as *B. mesentericus* and *B. megatherium*. Both peptonized milk without forming acid, both fermented dextrose and saccharose, *B. mesentericus* fermenting mannite as well. *B. bulgaricus* was still present.

November 10. The food was changed to fat, 3.1; sugar, 3.4; protein, 3.9, with the addition of four drachms of maltose.

November 12. The feces were alkaline to litmus paper. The suspension of feces in dextrose-free broth incubated twenty-four hours gave a very deep indol reaction. Milk tubes were coagulated and almost completely peptonized. Much gas formed in the fermentation tubes. Cocci were numerous, as were colon bacilli and *B. mesentericus* with many oval spores. *B. bulgaricus* was not found, though ripened milk was still used in the food.

November 16. The food was changed to the "synthetic" mixture, fat, 2.9; sugar, 1.5; protein, 5.8.

November 18. Cocci and colon bacilli were numerous. The feces were alkaline to litmus and a twenty-four hour culture in dextrose-free broth gave a deep

indol reaction. The most numerous variety of organism present was a gram-positive bacillus isolated as *B. mesentericus*. On gelatin plates many slowly liquefying colonies developed.

November 20. In the smears there were many spore-bearing gram-positive bacilli on a background of colon-like gram-negative bacilli; some cocci; some gram-positive bacilli without spores. In the fermentation tubes the gas was reduced to half the quantity of the previous days, and in the one containing lactose broth some acidophilus and very many bifidus bacilli grew together with *B. mesentericus* and cocci.

November 21. After a sharp rise in temperature and the development of some prostration, a laxative was given and the food changed to barley water for a day. The laxative brought away large numbers of the organisms present the day before, and more *B. acidophilus* and *B. bifidus*, and, in addition, *B. aerogenes capsulatus*.

During the next week the child was fed only diluted milk, varying from fat, 1.2; sugar, 5; protein, 1, to fat, 2.20; sugar, 5; protein, 1.75. The spore-bearing bacilli diminished in number but did not disappear entirely. *B. bifidus* remained in small numbers, and so did *B. aerogenes capsulatus*. Within another week, during which time the sugar in the food was kept at 4.50 per cent., *B. acidophilus* and *B. bifidus* had increased in number, and only an occasional spore-bearing bacillus was seen. The amount of gas diminished, and the milk-tubes were about half peptonized. The second observation was then begun on the same child.

Observation 2.—F. H., now 1 year old, Dec. 12, 1912. Food: Fat, 2; sugar, 5.7; protein, 2.6. Maltose in food. The cocci and the colon bacilli were numerous, but *B. acidophilus* and *B. aerogenes capsulatus* were more so. *B. megatherium* appeared in forty-eight hours (from the maltose). The amount of gas varied, but was rather large, and milk was almost completely peptonized.

December 26. "Synthetic" food begun: fat, 2.1; sugar, 1.7; protein, 6.3. During the next four days *B. mesentericus* appeared in large numbers, while *B. aerogenes* disappeared. The gelatin plates were rapidly liquefied. *B. acidophilus* was less numerous, *B. coli* more so.

December 30. Fever and prostration developed. Castor-oil was given, after which *B. bifidus* reappeared and increased markedly during the next four days, the food in that time being the milk formulas 1-5-90 and 2.60-5-2.40.

Jan. 4, 1912. Cocci, colon bacilli, *B. acidophilus*, *B. bifidus*, and an occasional *B. mesentericus*.

It will be noted that the change in the intestinal flora throughout these two observations was a very gradual one, consisting of a diminution but not a complete disappearance of the normal fermentative flora and its replacement by some proteolytic varieties. While the relative differences in the proportions of the amount of protein and carbohydrates in the food were as wide as it is possible to make them in the feeding of an infant of this age, it nevertheless follows that as long as the diet remains one composed entirely of milk ele-

ments, the absolute proportions cannot be changed to such a degree as to alter the flora completely. Thus the slow and incomplete change is accounted for. Compared with the results of the experiments of Herter and Kendall⁸ on kittens and monkeys, where the protein diet of meat and eggs was changed to one of milk plus dextrose, the difference is certainly a striking one. The presence of *B. acidophilus*, at a time when the reaction of the feces was neither amphoteric nor alkaline to litmus, may be accounted for by the fact that this acid-loving bacillus really grows better in a slightly alkaline medium, a fact pointed out by Rodella.⁹ Certainly *B. bifidus* branches very much more in acid than it does in alkaline media, which accounts for the straight forms seen in the stained smears from these stools, and proved to be *B. bifidus* by culture in the fermentation tubes containing sugar broth. It will also be observed that after the catharsis and a return to a diluted milk formula at the end of the periods of study, the child's intestinal flora was about the same as it had been at the beginning. The return to normal was practically accomplished in from seven to ten days.

Observation 3.—F. D., aged 4 months, Jan. 1, 1912. Food, milk with following percentages: fat, 2.40; sugar, .50; protein, 2.20. Gram-stained preparations showed the presence of cocci in pairs and chains, few colon-like bacilli and *B. acidophilus*. In cultures all these and *B. bifidus* appeared. Two days later *B. megatherium*, from the maltose used in the food, appeared in the feces. After a change to food made with ripened milk (1.7–2.20–3.5) *B. bulgaricus* was seen. With an increase of the protein in the food to 4.7 per cent. spore-bearing *B. mesentericus* appeared and the colon bacilli were numerous. *B. bifidus* was present in small numbers only. Gas production in the fermentation tubes was very marked, milk-tubes were peptonized and many liquefying colonies grew on gelatin plates. The "synthetic" food (2–1.50–6) was begun on January 22, and on the following day the colon forms were very numerous, but spore-bearing bacilli, together with acidophilus and bifidus, gave the smear a gram-positive appearance. The spores were of a variety of shapes. Some were oval with only a small cap of bacillus body left at either end; some were round and placed at one end of the bacillus, giving an appearance similar to that of the tetanus bacillus, and some were of the same diameter as the bacillus body, in which they lay nearer one end than the other. Still both acidophilus and bifidus continued to grow until the rise of temperature and accompanying prostration on January 26. Many of the bifidus bacilli were vacuolated, and the *B. mesentericus* showed many gram-negative, evidently degenerated elements. On February 2 the picture was practically as it had been at the beginning of the study, but a few oval spore-bearing bacilli still

⁹ Rodella, A.: *Centralbl. f. Bakteriol.*, 1901, xxix, 717.

remained. There was in general very little difference between this observation and the preceding ones.

Observation 4.—J. S., aged 7½ months. Food, milk with following percentage: fat, 1.45; sugar, 5; protein, 3.50. Feb. 27, 1912.

The preliminary examination of this child's rectal contents showed the presence of cocci in pairs and chains, comparatively few colon bacilli, some acidophilus and straight forms of bifidus, and very few spore-bearing bacilli probably taken in with the maltose added to the food. The picture was distinctly a gram-positive one. On the above food only a small amount of gas formed in the fermentation tubes, except on one day when, for no apparent reason, the gas formation was more than doubled and the comparative numbers of colon bacilli were greatly increased. Evidently peristalsis had brought down those bacilli from the region of the ileocecal valve just before the specimen was obtained for that day's observation. *B. bulgaricus* in small numbers appeared in the feces, but the cocci were by far the most numerous forms in both smears and cultures.

There was no appreciable change in the bacteria until the protein in the food reached 6 per cent. with 2.5 per cent. of sugar, when spore-bearing, gram-positive bacilli began to appear and *B. bulgaricus* was no longer found.

On this high protein food the floral changes proceeded slowly, cocci still remaining very numerous, colon bacilli gradually increasing in numbers, spore-bearing gram-positive bacilli increasing very slowly, and a few vacuolated forms of *B. bifidus* persisting.

March 18 the "synthetic food" began, fat, 2.1; sugar, 1.7; protein, 6.3, after which, although there were many free spores as well as those contained in both gram-positive and gram-negative bacilli, the numbers of acidophilus and bifidus bacilli were still so numerous as to grow without difficulty in lactose broth fermentation tubes and from them in deep stick lactose agar. After the febrile attack and the subsequent catharsis human milk was added to the infant's food. Within two days *B. bifidus* had appreciably increased in numbers, although *B. mesentericus* and spores were still present. On the third day *B. acidophilus* was isolated from the rectal contents. The return to a normal intestinal flora was more rapid in this child than in the other two. Whether the comparatively small amount of human milk (11 ounces) given him helped this event, or whether it was only a coincidence in a rather resistant baby, it is difficult to say without a repetition of the observation. It is suggestive, however, and agrees with Moro's¹⁰ experience, that when human milk is given to an artificially-fed baby, the physiologic flora appears in the stools on the second or third day.

Observation 5.—F. D., now 8 months old; same child as in Observation 3.

A preliminary study of specimens of the rectal contents showed the presence of cocci, colon bacilli, *B. acidophilus*, *B. aerogenes capsulatus*, and a few gram-positive bacilli containing oval spores.

May 1, 1912. Food: fat, 2.6; sugar, 4.5; protein, 5.0. Within three days the formula was changed to fat, 2.6; sugar, 2.5; protein, 6, and the gram-stained slides prepared from the fecal material showed a very mixed picture. There were gram-positive cocci, very long gram-positive bacilli, some vacuolated gram-positive bacilli in parallel pairs or in small groups, large gram-positive bacilli contain-

¹⁰ Moro, E.: Jahrb. f. Kinderh., 1905, lxi, 687.

ing spores, small ovals containing spores, gram-negative bacilli like the colon varieties, large gram-negative, granular bacilli, and some gram-negative bacilli containing spores. The amount of gas found in the fermentation tubes was very small, the milk tubes were coagulated but only very slightly peptonized. During the following week the picture remained the same, the bacteria cultivated from the feces including streptococci, staphylococci, *B. coli communis*, *B. bulgaricus*, and *B. mesentericus*. *B. bifidus* grew in the fermentation tubes. The only change observed during the following week was a decided increase in the amount of peptonization in the milk and gelatin cultures inoculated from the feces, and, in the smears, the number of spore-bearing bacilli increased comparatively, though all the other forms remained.

May 20. "Synthetic" food, fat, 2.06; sugar, 1.8; protein, 6. For two days there was practically no change distinguishable. At the end of that time, although the reaction of his rectal contents remained distinctly acid to litmus paper, the spore-bearing bacilli increased somewhat in numbers, *B. bulgaricus* had disappeared, the colon bacilli were less numerous, and bifidus forms were very few.

May 23. On the third day a rise in temperature occurred and the carbohydrate content of the food was increased to 5 per cent. the following morning, the protein remaining at 6 per cent. The spore-bearing gram-positive bacilli, isolated as *B. mesentericus*, still dominated the picture, bifidus forms also being present. As the temperature continued high, castor-oil was administered on May 25.

For a period of twenty days this child was fed on a mixture made with ripened milk, and *B. bulgaricus* was present in his rectal contents throughout that time. In one of the other studies (Observation 1) the period in which ripened milk was used in the food lasted twenty-three days, and in Observation 4 it lasted eighteen days. In neither of these cases, however, did the acid reaction of the rectal contents continue until the appearance of the fever, nor did *B. bulgaricus* appear in the feces throughout the entire period of feeding with ripened milk. This prolonged period of acidity did seem to delay the increase of proteolytic bacteria, though it did not entirely prevent their development. It would also seem that since all the clinical symptoms accompanying the end of this observation were as marked as they had been in the other four, the proteolytic bacteria were not the cause of those symptoms, but only the inevitable result of a comparatively high protein content in the food, and not, in themselves, harmful.

The slow increase of the proteolytes and the persistence of *B. bifidus* would seem to exemplify the statement of Tissier¹¹ that the rôle of *B. bifidus* is to keep the intestinal tube acid enough to arrest

¹¹ Tissier, H.: Ann. de l'inst. Pasteur, 1905, xix, 119 and 272.

the development of many abnormal invaders, and to remove the proteoses necessary to increase the acidity, but even the combined acidity of the two was insufficient to prevent *B. mesentericus*, which attacks all proteins, from developing.

SUMMARY.

To sum up the results of these five observations, we find that the varieties of bacteria identified as present in the rectal contents of the five children before and during the period of study included the following: *Albococcus*, *Micrococcus ovalis* or *Enterococcus*, a streptococcus proteolyzing milk, a streptococcus not proteolyzing milk, *B. coli communis*; *B. coli communior*; *B. lactis aerogenes*; *B. acidophilus*; *B. bifidus*; *B. bulgaricus*; *B. fluorescens*, liquefying gelatin. *B. dysenteriae* (Shiga) was found in one case.

During the periods of high carbohydrate in the food the cocci, colon bacilli, acidophilus and bifidus bacilli were most numerous; sometimes *B. aerogenes capsulatus* appeared.

During the periods of high protein content in the food, *B. mesentericus* gained the ascendancy, *B. aerogenes capsulatus* sometimes persisted, and the colon bacilli were increased in numbers. But the acidophilus and bifidus bacilli did not entirely disappear, probably because the change in the food was not complete nor prolonged enough.

Bacteria introduced with the food (*B. fluorescens*, *B. bulgaricus*, *B. subtilis*, *B. megatherium*) were found in the feces within a period varying from twenty-four to seventy-two hours.

The transition in the flora from one period to another was always gradual, never abrupt, recognizable changes requiring forty-eight to seventy-two hours to develop.

The return to the bacterial picture present at the beginning of the observation, which may be assumed to be the normal for that particular infant, was accomplished in from seven to ten days after the end of the study and subsequent catharsis.

As regards the children studied a second time, neither one gave any evidence of a more rapid reaction during the second observation than he had shown during the first.

PART IV. OBSERVATIONS ON METABOLISM.

By ANGELIA M. COURTNEY.

(Fellow of the Rockefeller Institute.)

ASSISTED BY

JESSIE A. MOORE.

(New York.)

METHODS OF ANALYSIS.

The methods used were mainly those described in preceding papers.¹² A few changes and additions are to be noted. In the determination of feces fat, a third portion of dried material was mixed with an excess of alcoholic solution of N/5 sodic hydrate, thus binding the free fatty acids and leaving only neutral fat to be extracted. The values thus obtained for neutral fat are subtracted from those obtained from the simple extraction of the dried material, giving values for the free fatty acids present. Total ash was determined by heating in a platinum dish with small amounts of ammonium nitrate, cooling and weighing until weight is constant. For the determination of chlorid, the solid material was thoroughly mixed with dry sodium carbonate and charred, the alkali neutralized by nitric acid, the solution filtered off and titrated according to the Volhard method. Chlorids in the urine were also determined according to Volhard. The method for determining calcium has been described. Magnesium was determined in the filtrate remaining from the calcium determination by precipitating ammonium magnesium phosphate by addition of disodium phosphate, filtering through a Gooch crucible after an interval of several hours and heating the precipitate with a blast lamp until it is converted to magnesium pyrophosphate. Phosphates were determined in the urine by titration with uranium nitrate, potassium ferrocyanid being used as indicator.¹³ The solid material, dried milk and feces, was ashed to whiteness in a platinum crucible with powdered magnesium oxid, the residue dissolved in 10 c.c. hot 5 per cent. hydrochloric acid, neutralized to phenolphthalein with 5 per cent. sodic hydrate and 0.5 to 1 c.c. excess added, again neutralized by 10 per cent. acetic acid containing 10 per cent. sodium acetate and 2 to 3 c.c. more acetate solution added.¹⁴ The solution was then titrated with uranium nitrate as in case of urine.

PREPARATION OF FOOD MIXTURES.

"*Synthetic*" Food Mixture.—A casein curd was obtained by allowing junket tablets to act on 4,000 c.c. of fat-free milk. The curd was drained through cheese cloth and washed four times with boiled water. A solution of the casein was effected in the following manner: The moist curd was ground in a mortar with

¹² AM. JOUR. DIS. CHILD., 1911, i, 321-340; January, 1912, iii, 1-4.

¹³ AM. VOZÁRIK, Ztschr. f. Phys. Chem., 1911-12, No. 76, v and vi, 426 and 433.

successive small quantities of N/10 sodic hydrate and hot water until the mass resembled a thick boiled starch. (The alkali and water were sterilized by boiling.) A clear fluid solution of the casein was then effected by heating the paste for a few minutes in a double boiler. The solution of casein did not react alkaline, although it required 250 c.c. of sodic hydrate (1 gm. of the salt) to accomplish the solution. The final food mixture was made up by adding to the casein solution 200 c.c. of milk and the desired quantity of 32 per cent. cream. Boiled water was added to make up a volume of 1,500 c.c.; of these 1,260 c.c. were employed for feeding and the remaining 240 c.c. for analysis.

Other High Protein Food Mixtures.—The casein curd was prepared in the manner described in the preceding paragraph. The curd was then ground up and pressed through a fine sieve by the aid of 600 c.c. of ripened milk containing 2 per cent. fat. To this suspension boiled water, lactose, and 32 per cent. cream were added in desired proportion. The amount of fat-free milk employed for the curd, of lactose, and of cream varied for each feeding period in accordance with the desired formula; the quantity of ripened milk remained constant. The amount of protein in each mixture was calculated on the basis of average values obtained from the analysis of several samples of the curd obtained in the manner described in the preceding paragraph.

CASE 1.—Francis H. There were ten periods of observation beginning September 14, 1911, and ending January 1, 1912; each period represented a change in the food. During the first two he was on a normally-balanced diet the aim of which was to provide a standard for comparison with the other diets in which one of the constituents predominated, and the other two were given in a minimum quantity. The percentage composition of the food during the first period of nineteen days was 2.6 fat, 5.0 carbohydrate, and 2.4 protein; of the second standard period of twelve days, 2.6 fat, 3.0 carbohydrate, and 2.4 protein (*vide* Table 1). The daily caloric intake of the food mixture of the first period contained 721 calories, and of the second period 690 calories. The general condition of the infant on this diet was fairly satisfactory. The average gain in weight was about 12 gm. per day in the first period, and about 5 gm. per day in the second period. The stools were very watery, contained some curds, were foamy, greenish and contained some mucus. The nitrogen balance was positive, reaching 1.2 gm. per day. The general composition of the urine indicated only a slight deviation from the normal in the first period, and scarcely any in the second period. The percentage of ammonia nitrogen in the urine was 8.5 of the total in the first period, indicating, perhaps, very slight acidosis. Creatin and creatinin output showed values differing little from those of the following periods. The ash retention in the first period was considerably higher than in the second, reaching the value of 2 gm. per day in the first, and 0.3 gm. in the second period. The stools presented a comparatively high total acidity, reaching in the first period the value of 903 and in the second period 779 per 100 grams of dried material.

In the third period lasting four days, and the fourth period lasting five days, the fat was removed from the food mixture in the third, and lowered to the proportion of 1.6 per cent. of the total food intake in the fourth period. The caloric intake was 567 calories in the third, and 649 calories in the fourth period. On

this diet the appearance of the stools changed considerably. The color was copper-yellow, of watery appearance with considerable mucus, and with many hard curds. In the fourth period they gradually became granular, with many small fat curds, and the quantity of mucus then diminished. The general condition of the infant, as indicated by the body weight of the patient, was better in the third period than in the fourth, showing a gain of 25.7 gm. per day in the third, and a loss of 23.3 gm. per day in the fourth period. The nitrogen balance was positive in both periods, being 1 gm. per day in the third, and 0.7 gm. per day in the fourth. The composition of the urine in both periods indicates less acidosis than one noticed in the first two periods. Very striking were the fluctuations in creatin output; while the output of creatinin remained nearly constant.

This observation regarding creatin is rather in conflict with those made by Rose¹⁴ in Professor Mendel's laboratory and by Folin.¹⁵ It is not possible at the present moment to give an adequate explanation of the differences in the observations of Rose and of Folin on one hand and ours on the other. The considerable difference in the ages of the children may contain the reason for the discrepancy, or it may be occasioned by some accident of the observation.

The total mineral balance was less favorable during these two periods as compared with the first two. The chemical changes in the composition of the feces were not striking, the acid value per 100 grams being about the same as in the second period. As a significant distinction one may point out the proportion of soaps in the stools, which presented a much lower value than in the preceding periods.

To sum up, a food mixture composed of a high proportion of carbohydrate and low in fat resulted in a change in the general appearance of the stools, and in a fall of the mineral balance. There was noted also a marked change in the creatin output. The importance of the creatin values cannot be discussed at the present moment.

In the observations following the fourth period we aimed to change gradually the diet so as to raise principally the protein intake and to lower the fat and the carbohydrate. The highest protein intake was 6 per cent. of protein on the total volume of the food mixture.

During these periods the condition of the infant, as expressed by the daily gain in weight, was improved. The gain was 55 gm. per day the fifth, 64.8 gm. in the sixth, and 10.5 gm. per day in the seventh period.

There was little change in the composition of the urine (Table 2) which could be considered of significance; perhaps worthy of note is again the creatin output, which still remained high; in fact, it reached its highest value during the fifth period. During that period the total calorific intake was lower than that of any of the preceding periods. In the following period the calorific intake was raised to 900 from 500, and with this change there was noted a fall in the creatin and simultaneously a rise in the creatinin output. During the following period in which the protein intake was raised to a still higher level, and the carbohydrate intake slightly increased, there was noted a still greater fall in the creatin and a still higher rise in the creatinin output. Thus it seems that in this infant the

¹⁴ Mendel, L. B., and Rose, W. C.: *Jour. Biol. Chem.*, 1911, x, 213, 265.

¹⁵ Folin, Otto, and Denis, W.: *Jour. Biol. Chem.*, 1912, xi, 3, 253.

creatin output diminished with the increase of the protein intake. The proportion of carbohydrate in the food apparently had less influence on the output of creatin and creatinin.

However, the high protein intake caused a very striking change in the character of the stools (Table 3). They became for the most part pasty, grayish-yellow, smooth; in other words, practically normal. The total acidity showed a marked fall from 900 in the first period to 148 in the seventh period. On the contrary, the proportion of soaps was greatly increased, and with it followed an increased mineral retention.

The nitrogen retention during these periods reached its highest values. It is worthy of note, that the very high retention did not last more than one or two days after the beginning of the very high protein intake, and reached its equilibrium generally on the third day.

The most significant is the eighth period of observation (November 16 to November 21). The food at that period was so changed as to contain the same or a slightly higher proportion of protein, as in the previous period, with a lower intake of carbohydrate and fat, so as to bring down the average daily calorific intake to 632 calories. On this diet the infant seemed to remain in a normal condition for three days. On the fourth day, he developed high temperature, and other symptoms which are described in the clinical part of this communication. From the standpoint of chemical metabolism, this period presented nothing significant that could distinguish it from the preceding period. Everything that was said about the peculiarities of the urine, stools, and general balance of the preceding period applies also to this period.

The lower average values for the balances of nitrogen and other constituents are principally due to the fact that the child refused food during the days of high fever.

The only very striking peculiarity of this period was the *retention of chlorids* beginning a few days immediately preceding the fever and continuing during the days following it.

In the following ninth period (December 12 to December 23) a change of the diet was again made in the way of diminishing the protein to a minimum and raising the carbohydrate to a maximum. During this period the condition of the child and the character of the stools and urine were practically the same as during the third period of observation, when the carbohydrate intake was at its maximum.

The food of this period differed from that of the preceding periods not only in the proportion of fat, protein, and carbohydrate, but also in containing for part of the time sodium phosphate, which was added to the daily food mixture. However, this was without any effect on the body temperature.

The tenth period (December 26 to January 1) was planned to be a repetition of the observations made in the eighth period, and the food was made up in exactly the same way. This food is referred to as the "synthetic" food. Under the influence of that food the child remained in perfectly normal condition for five days, and on the sixth day he again developed a marked rise in body temperature which lasted until the following day.

Again there was observed a retention of chlorids beginning a few days preceding the fever and lasting after the body temperature was restored to normal.

CASE 2.—The observations were made on Jacob S., aged 7½ months. The observations on this infant and also on Case 3 are divided into periods corresponding to food changes. These changes were planned to furnish definite evidence as to whether or not the "synthetic" food mixture was capable of disturbing the normal body temperature. With this in view the food intake was gradually modified so as to preserve approximately the same calorific intake by replacing the carbohydrate with protein until the maximum protein intake of 6 per cent. of the food mixture was reached.

The general changes in the composition of the urine and stools had the same character as in the periods of high protein intake of the first infant, namely, there was noted a gradual fall in the acidity of the stools and with it a rise in the proportion of soaps and general increase in the mineral balance. The nitrogen retention steadily increased until the maximum protein intake was reached. The child was gaining in weight continually, though slowly, and the total gain was about 350 gm.

When the highest protein intake of 6 per cent. per day was reached, the food was again changed to the one designated as "synthetic" mixture. On the fourth day of that régime the infant developed fever reaching 103.2° F. This fever continued for seven days until the food was changed. The general character of the stools, urine, and general metabolism presented nothing of significance that distinguished the period of "synthetic" diet from the immediately preceding period. Typical only was the retention of chlorids as noted in both observations on Case 1. The changes that are recorded in the last days of this period were not due to the character of the food, but to the administration of calomel and castor-oil, which caused free catharsis.

Two series of observations were made on Case 3, Francis D. The first one aimed simply to determine the capacity of the "synthetic" food mixture to cause a rise in body temperature (Table 5). There was no investigation made into the chemical metabolic changes. The results of this observation were in all respects confirmatory of the first three observations made on the other infants. Fever developed on the fourth day.

The second series of observations made on this infant was planned principally to ascertain whether the influence of the "synthetic" food mixture on the body temperature is a constant occurrence; during this period clinical observations were accompanied by a study of the chemical metabolism. Again on this occasion the changes of the diet aimed principally at raising the protein intake to a maximum of 6 per cent. of the milk formula. The food mixture contained 5 per cent. of protein when the infant was first placed in the metabolism bed. In the course of four days the maximum of 6 per cent. was reached, and this was continued for sixteen days. There was a constant gain in weight and a constant rise in the nitrogen retention. The general changes in the composition of the urine and feces were the same as in the other experiments. The most striking features were the fall in the total acidity of the feces, the rise in the proportion of soap, and the rise in the mineral balance.

After the child had been for sixteen days on the maximum protein intake, the diet was again changed to the "synthetic" food mixture. On the fourth day after this change there was again a marked rise of the body temperature which reached

on the fifth day of the period 104° F. The addition of lactose, raising the carbohydrate to 5 per cent., had no effect on the temperature, though it was continued for two days. The condition of the infant, however, did not permit of prolonging the observation and on the seventh day of this period the "synthetic" food was discontinued. The general character of the urine and stools during this period did not present any striking differences from that of the previous ones, and those differences that did appear may be explained by looseness of the bowels produced by prolonged administration of milk of magnesia. There was again noted the usual retention of chlorids.

TABLE 2.
AVERAGE DAILY FOOD INTAKE.
Case 1, Francis H.

Period.	Dates.	Composition of Food.	Volume In- take, c.c.	Fat, gm.	Carbohy- drate, gm.	Protein, gm.	Nitrogen, gm.	Total Number Calories.	Chlorides as NaCl, gm.	Phosphates as P ₂ O ₅ , gm.	Total Ash as Oxides, gm.	CaO, gm.
1	Sept. 14 to Oct. 3	Sixty-five per cent. milk, 5 per cent. lime-water, barley water to volume, making fat 2.6 per cent., protein 2.4 per cent.; lactose added to make 5 per cent.	1,255	32.4	57.0	29.8	4.76	721	1.68	2.58	7.46	2.55
2	Oct. 4-14	Like that of Period 1, except that no lactose added, making carbohydrate 3 per cent.	1,260	33.6	48.5	27.4	4.54	690	1.68*	2.55	7.60	3.21
3	Oct. 16-19	Fat-free milk, cane-sugar added to make total carbo- hydrate 6 per cent.; protein 3.5 per cent.	1,260	0	75.5	41.3	6.61	567	1.57	2.77	9.86	3.65
4	Oct. 20-24	Mixture of fat-free and full milk, making 1.6 per cent. fat and 3.0 per cent. protein; cane-sugar to make 4.7 per cent. carbohydrate; 10 per cent. barley-water, 5 per cent. lime-water included.	1,260	20.0	59.6	34.8	5.57	649	1.88	2.40	8.88	3.01
5	Oct. 25-29	Three-fourths ordinary protein milk, $\frac{1}{4}$ barley-water, making fat 1.9 per cent., carbohydrate 2.3 per cent., protein 2.9 per cent.	1,230	23.0	28.0	35.7	5.71	504	1.26	2.21	7.71	2.80
6	Oct. 31 to Nov. 6	Protein milk made from casein of 2 liters full milk, 1 liter ripened fat-free milk and 120 c.c. water, making fat 3.6 per cent., carbohydrate 3.1 per cent., protein 5.6 per cent.	1,243	44.2	38.7	69.5	11.12	913	2.23	4.0	13.13	2.52
7	Nov. 14-16	Protein milk made from casein of 1 liter full milk, 1 liter ripened fat-free milk and 120 c.c. water, 4 drams maltose to day's food, making fat 3.5 per cent., carbohydrate 3.4 per cent., protein 3.9 per cent.	1,260	43.5	42.7	49.6	7.94	845	1.81	3.14*	10.28	2.72
8	Nov. 16-21	So-called synthetic food, fat 2.9 per cent., carbo- hydrate 1.5 per cent., protein 5.8 per cent. For description see text.	1,260	31.8	18.5	71.6	11.46	705	.730†	3.60†	11.70	3.37†
			1,260	36.6	18.9	65.4	10.44	722	10.58
			1,260	39.7	20.3	78.0	12.48	815	11.62
			1,135	33.8	17.3	68.4	10.94	702	9.85
			580	15.7	8.6	36.8	5.9	355	5.47

* Estimated.

† Average.

Case 1, Francis H.—Continued.

		Mixture fat-free and full milk, making fat 2 per cent., protein 2.5 per cent.; dextrimaltose added to make carbohydrate 6.4 per cent.; 20 per cent. barley-water and 5 per cent. lime-water included. In part of period (Dec. 16-20) 8.3 gm. mono- and disodium phosphate added to day's food..... So-called synthetic food, fat 2.1 per cent., carbohydrate 1.7 per cent., protein 6.3 per cent.....	Daily Figures									
9	Dec. 12-16 16-20 20-23		1,200	26.2	72.4	31.0	4.96	732	1.59†	2.47†	6.99	1.56†
			1,260	20.6	85.0	31.3	5.0	728	14.94
			1,260	29.9	85.9	31.0	4.96	818	8.91
10	Dec. 26 to Jan. 1		515	10.6	8.6	31.2	4.99	480†	.58††	2.90†	4.78	3.18†
			1,045	27.9	18.7	63.7	10.19	7.45
			1,135	26.1	19.6	72.7	11.63	10.49
			1,184	23.8	19.6	82.5	13.20	9.94
			1,230	23.5	20.4	78.2	12.50	11.78
			180	12.1	1.94	1.61

Case 2, Jacob S.

1	Feb. 27-29	Mixture fat-free and full milk to make fat 1.45 per cent., protein 3.5 per cent., lactose added to make 5 per cent.; 2 dr. neutral maltose and $3\frac{1}{2}$ dr. olive oil added to day's food.	1,050	26.6	52.2	33.7	5.39	676	1.13	2.25	5.41	1.98
2	Feb. 29 to March 4	Fat 1.45 per cent., carbohydrate 4.5 per cent., protein 4 per cent., $3\frac{1}{2}$ dr. olive oil added to day's food. For method of making up this formula and the four following see text.	998	27.1	45.6	40.7	6.50	637	1.01	2.53	5.26	2.12
3	March 4-7	Fat 1.45 per cent., carbohydrate 4.0 per cent., protein 4.5 per cent., $3\frac{1}{2}$ dr. olive oil added to day's food.	1,007	29.1	40.5	42.6	6.82	647	1.19	2.48	5.47	2.28
4	March 7-10	Fat 1.45 per cent., carbohydrate 3.5 per cent., protein 5.0 per cent., $3\frac{1}{2}$ dr. olive oil added to day's food.	1,015	24.9	37.8	51.9	8.31	636	1.40	2.99	6.45	2.37
5	March 10-13	Fat 1.45 per cent., carbohydrate 3.0 per cent., protein 5.5 per cent., lime-water 5 per cent., $3\frac{1}{2}$ dr. olive oil in day's food.	1,025	24.3	32.8	57.0	9.12	628	1.23	3.22	6.66	2.74
6	March 13-18	Fat 1.45 per cent., carbohydrate 2.1 per cent., protein 6.0 per cent., lime-water 5 per cent., $1\frac{1}{2}$ dr. olive oil in day's food.	1,030	16.7	28.6	61.6	9.85	563	1.19	3.64	7.97	3.35

‡ Estimated. † Average.

Case 2, Jacob S.—Continued.

Period.	Dates.	Composition of Food.	Volume In- take, c.c.	Fat, gm.	Carbohy- drate, gm.	Protein, gm.	Nitrogen, gm.	Total Number Calories.	Chlorides as NaCl, gm.	Phosphates as P ₂ O ₅ , gm.	Total Ash as Oxide, gm.	CaO, gm.
7	March 18-27	So-called synthetic food, fat 1.45 per cent., carbo- hydrate 1.8 per cent., protein 6.0 per cent., 1½ dr. olive oil added to day's food; 120 c.c. breast milk March 25 and 210 c.c. March 26.....	900 1,050 975 630 960 780 1,050 930 725	14.7 15.2 17.1 14.2 16.2 14.3 14.1 14.9 14.8	13.8 19.8 19.0 11.8 19.2 15.3 19.8 23.9 24.9	47.9 66.0 59.9 35.8 66.4 53.2 65.6 50.4 35.2	7.67 10.56 9.66 5.74 9.68 8.51 10.50 8.07 5.04	397 502 491 333 486 421 490 448 386	.327 .574 .515 .242 .463 .434 .642 .400 .300	2.49 3.44 2.98 1.98 3.22 2.66 2.82 2.74 1.90	6.13 7.66 7.54 4.34 6.66 6.27 7.75 6.29 4.08	2.77 2.77 3.44 2.08 2.93 2.66 3.76 2.62 1.69

Case 3, Francis D.

1	May 1	Fat 2.6 per cent., carbohydrate 4.5 per cent., protein 5.0 per cent., 2 dr. olive oil added to day's food..	1,260	38.2	65.8	63.4	10.13	917	1.44	3.84	7.65	3.24
2	May 2-4	Fat 2.6 per cent., carbohydrate 3.5 per cent., protein 5.5 per cent., 2 dr. olive oil in day's food.....	1,260	38.0	46.0	71.4	11.42	877	1.49	4.12	9.34	3.71
3	May 4-19	Fat 2.6 per cent., carbohydrate 2.5 per cent., protein 6.0 per cent., 2 dr. olive oil in day's food. For method of making up this and two preceding formulae see text.....	1,253 1,260 1,260 1,260 1,260	38.6 36.2 41.4 40.2 37.5	33.8 22.0 22.0 21.9 21.8	74.9 73.4 74.8 71.2 76.5	11.98 11.75 11.95 11.40 12.24	846 739 794 767 764	1.47 .253 .360 .256 .359	4.23 3.75 3.95 3.62 1.13	10.50 8.65 9.95 9.11 9.60	4.12 3.36 4.17 3.69 3.85
4	May 20-24	So-called synthetic food, fat 2.6 per cent., carbo- hydrate 1.8 per cent., protein 6.0 per cent., 2 dr. olive oil in day's food; 36 gm. lactose added to day's food May 23-24.....	1,260	36.4	58.2	69.0	11.06	863	1.67	3.61	7.99	3.39

TABLE 3.
URINE CONSTITUENTS. AVERAGE DAILY VALUES.
Case 1, Francis H.

Period.	Dates.	Volume, c.c.	Specific Gravity.	Indican.	Total Nitrogen, gm.	Ammonia Nitro- gen, gm.	Percent Total N.	Kreatinin Nitro- gen, gm.	Kreatinin Nitrogen, gm.	Inorg. Sulphates as H ₂ SO ₄ , gm.	Etheral Sulphates as H ₂ SO ₄ , gm.	Total Sulphates as H ₂ SO ₄ , gm.	Chlorides as NaCl, gm.	P ₂ O ₅ , gm. Phosphates as	Total Ash as Oxids, gm.	CaO, gm.
1	Sept. 14 to Oct. 3	426	1.010 1/2	Gen. Negative	2.81	.240	8.5	.063	.023	.362	.123	.485	.817	.587	1.44	.079
2	Oct. 4-14	541	1.011	Neg. to High 10+	3.18	.118	3.7	.055	.011	.472	.131	.603	1.179	.635	2.72
3	Oct. 16-19	547	1.013	Negative	4.66	.152	3.2	.043	.047	.722	.122	.844	1.549	.731	3.39
4	Oct. 20-24	663	1.011	Negative	4.29	.073	1.7	.050	.078	.772	.080	.852	1.894	.729	4.45	.673
5	Oct. 25-29	703	1.011	Faint trace or neg.	4.78	.132	2.8	.042	.108	.695	.139	.834	1.791	.577	2.48
6	Oct. 31 to Nov. 6	488	1.018	Neg. or small trace	7.45	.342	4.6	.064	.055	1.078	.133	1.211	1.392	.832	3.21	1.124
7	Nov. 14-16	475	1.016	Nearly negative	5.36	.158	3.0	.075	.008	.747	.188	.935	.806	.612	2.84
8	Nov. 16-21	550	1.012	Faint trace	5.30	.137	2.6	.071	.025	.671	.219	.890	.323	.636	1.69
		490	1.016	Faint trace	7.52	.178	2.4	.070	.059	1.129	.040	1.169	0	.639	1.65
		530	1.016	Trace	8.76	.279	3.2	.074	.008	1.122	.172	1.294	0	.780	1.59
		475	1.018	8-10	9.34	.326	3.4	.070	.025	.798	.558	1.356	0	.722	1.33
		350	1.019	16-20	7.36	.340	4.6	.081	.004	1.22	.105	1.385	0	.717	1.44
9	Dec. 12-16	514*	1.013*	Negative	3.35	.189	5.7	.043	.020	.523	.087	.610	1.53	.516	3.45
	16-20	3.08	.187	5.7	.041	.008	.492	.055	.547	1.55	.757	4.31
	20-23	3.70	.110	3.0	.044	.010	.538	.080	.618	1.98	.582	4.07
10	Dec. 26 to Jan. 1	255	1.021*	Negative	6.65	.337	5.1	.055	.023	.904	.062	.966	.292	.747	1.77
		330	Large trace	7.98	.344	4.3	.043	.020	.982	.019	1.001	.066	.525	1.52
		430	Trace	9.10	.327	3.6	.044	0	1.185	.017	1.202	0	.665	1.31
		410	Faint trace	8.80	.405	4.6	.054	.027	1.124	.011	1.135	0	.642	1.72
		390	8-10	7.10	†	..	.047	..	1.185	.094	1.279	0	.686	1.77
		355	Trace												

* Average. † Alkaline.

Case 2, Jacob S.

1	Feb. 27-29	493	1.013½	Small trace to neg.	2.83	.070	2.5	.041	.038	.456	.051	.507	.870	.578	3.24	.018
2	Feb. 29 to March 4	460	1.013	Neg. very high or faint trace	4.38	.146	3.3	.044	.018	.602	.085	.687	.907	.570	1.97	.042
3	March 4-7	410	1.014	Neg. to faint trace	4.80	.166	3.5	.043	.019	.741	.044	.785	.907	.558	1.61	.025
4	March 7-10	418	1.016½	Neg. or small trace	5.86	.346	5.6	.038	.025	.889	.055	.944	.843	.670	2.03	.018
5	March 10-13	458	1.019	Gen. consid. 5-20	6.44	.198	3.1	.034	.027	.969	.069	1.038	1.053	.704	1.63	.030
6	March 13-18	473	1.018	Trace to high 10-15	7.66	.395	4.0	.029	.046	1.157	.070	1.436	1.116	.752	2.06	.049
7	March 18-27	435	1.014½	Consid. 5	6.00	.246	3.8	.038	.033	.834	.106	.940	.721	.597	1.60
		445	1.018	Consid. 5	6.32	.133	2.1923	.114	1.037	.297	.651
		490	1.019	High 20-30	7.83	.187	2.4	.043	.031	1.063	.068	1.131	.348	.756	1.65
		390	1.018½	Consid. 5	6.42	.207	3.2	.046	.032	.952	.091	1.043	.462	.611	1.46
		230	1.023	Consid. 5	5.24	.187	3.6	.048	.042	.768	.021	.789	.191	.650	1.02
		270	1.022½	Small trace	6.25	.215	3.4	.055	.045	.907	.038	.945	.080	.622	1.05	.024
		370	1.015	Faint trace	5.17	.151	2.9	.051	.027	.752	.037	.789	.077	.416	.908
		345	1.016½	Faint trace	5.69	.182	3.2	.048	.036	.819	.095	.914	.092	.438	.973
		250	1.019	Negative	4.85	.186	3.8	.045	.034	.664	.049	.713	0	.547	.593

Case 3, Francis D.

1	May 1	440	1.017	Negative	6.38	.287	4.5	.035	.037	.965	.050	1.014	.573	1.433	2.53	.101
2	May 2-4	395	1.021	Negative	7.54	.381	5.1	.034	.036	1.151	.059	1.209	.888	1.53	2.84	.102
3	May 4-19	388	1.022	Mostly neg. or faint trace	7.87	.359	4.6	.031	.032	1.177	.055	1.232	.774	1.32	2.53	.111
4	May 20-24	340	1.023	5	7.65	.285	3.7	.039	.030	1.111	.060	1.170	.179	1.13	2.16
		260	1.022½	Faint trace	4.79	.123	2.6	.027	.028	.712	.043	.755	.015	.753	1.46	.018
		395	1.018½	Considerable trace	7.53	.236	3.1	.032	.017	1.105	.067	1.172	0	1.12	2.01
		470	1.018	Faint trace	8.46	.263	3.1	.033	.017	1.270	.055	1.325	0	1.36	2.51
		380	1.021	Considerable trace	7.37	.268	3.6	.043	.004	1.114	.109	1.223	0	1.09	2.13

Indican.—The figures in this column indicate color percentages; undiluted Fehling's solution being taken as 100. It will be noted that in Case 1 during the first observation, there was no excess of indican until the fourth day of the "synthetic" food; in the second period of observation there was no excess until the fifth day of the same food. In Case 2 the indican was variable before the "synthetic" period, sometimes being quite high. The highest point was reached on the third day of "synthetic" food while marked constipation was present. In Case 3 it was never in excessive amount during the long period antecedent to "synthetic" food, but it was high during that period. In the second period of observation only traces of indican were present at any time. *Sugar*.—Tests for sugar were made only occasionally, as when the specific gravity was high or the sugar intake considerably increased. In no case was sugar found in the urine. *Albumin*.—Daily examinations were made in all cases. In Cases 1 and 2 it was invariably absent. In Case 3 a considerable trace was noted but once, in the fifth day of the "synthetic" food.

DESCRIPTION OF FECES.

Case 1, Francis H.

1. Sept. 14-Oct. 3.—Thin, generally very watery, containing fat curds and few small hard curds, greenish-yellow, sometimes foamy; 8-12 stools daily.
2. Oct. 4-14.—Becoming more solid than in Period 1, more finely granular; 7-9 stools daily.
3. Oct. 16-19.—Orange-yellow, pasty or watery, containing hard curds and considerable mucus; 11-13 stools daily.
4. Oct. 20-24.—Watery or pasty, smooth and finely granular, containing small fat and protein curds, less mucus than in Period 3; 8-10 stools daily.
5. Oct. 25-29.—Mostly formed but soft, yellow-gray, smooth; 3-4 stools daily.
6. Oct. 31-Nov. 6.—Mostly formed, smooth, harder than in Period 5, portions watery or pasty, granular; 4-6 stools daily.
7. Nov. 14-16.—About as in Period 6; 4-5 stools daily.
8. Nov. 16-21.—After first day formed, hard and dry, containing fragments of hard curds; 3-6 stools daily.
9. Dec. 12-23.—Pasty or watery, containing fat curds, hard curds, and mucus, acid and rancid; 4-10 stools daily; more solid during last three days with less mucus.
10. Dec. 26-Jan. 1.—At first formed and semi-formed, rather soft and mealy, becoming hard and dry. After castor-oil was given largely watery and pasty, with small hard curds; 4-7 stools daily.

Case 2, Jacob S.

1. Feb. 27-29.—Pasty, slightly formed, partly fluid, granular with fat curds, partly greenish; 4 stools daily.
2. Feb. 29-March 4.—Mostly formed, yellow-gray, smooth; 1-5 stools daily.
3. March 4-7.—More watery than in Period 2, foul, partly greenish, small part formed; 2-5 stools daily.
4. March 7-10.—As in preceding period, but generally more formed; 2-3 stools daily.
5. March 10-13.—Formed and hard; foul; 3-6 stools daily.
6. March 13-18.—As in preceding period, often with traces of blood; 3-7 stools daily.
7. March 18-27.—As in preceding period; 2-4 stools daily, until after castor-oil was given, then thin, pasty and watery, containing fragments of hard curds and mucus, generally greenish; 7-8 stools daily.

Case 3, Francis D.

1. May 1.—Formed, smooth, portion dry and hard, larger part soft, yellow-gray; 4 stools.
2. May 2-4.—As in Period 1; 1-4 stools daily.
3. May 4-19.—For first six days as in Periods 1 and 2, after that larger part thinner and less formed; 1-4 stools daily.
4. May 20-24.—Mostly watery and pasty, containing hard curds and often mucus, in increasing amount, partly greenish; about 3 stools daily.

TABLE 4
COMPOSITION OF FECES. AVERAGE DAILY VALUES.
Case 1, Francis H.

Period.	Dates.	Dried Weight, gm.	Dried Weight, per cent. of Moist.	Water in Feces, c.c.	Total Nitrogen, gm.	% Dried Nitrogen.	Chlorid as NaCl, gm.	Phosphates as P ₂ O ₅ , gm.	Total Ash as Oxids, gm.	Total Ash, % of Dried Weight.	CaO, gm.	Total Fat, gm.	Total Fat, % Dried Weight.	Neutral Fat, gm.	Neutral Fat, per cent. Total Fat.	Free Fatty Acids, gm.	Fatty Acids, per cent. Total Fat.	Soap Fat, gm.	Soap Fat, per cent. Total Fat.	Total Acidity for N/10 NaOH, 24 hours c.c.	Total Acidity per 100 gm. Dried Wt.	Volatile Acids, 24 Hours, c.c. N/10 NaOH.	Volatile Acids per 100 gm. Dried Wt.
1	Sept. 14 to Oct. 3	10.9	7.8	251	.782	4.0	.09	.62	3.08	20.0	1.28	6.96	34.7	4.72	66.2	1.18	16.8	1.06	17.0	181.9	903	118.6	636
2	Oct. 4-14	15.4	8.0	191	.707	4.6	.49	.80	4.56	29.6	1.34	4.61	29.5	2.94	63.6	.80	16.1	.87	20.3	119.9	779	137.3	907
3	Oct. 16-19	16.8	8.4	195	.922	5.5	.19	1.53	5.93	35.4	2.33	.43	3.9	.43	100.	133.3	799	110.0	658
4	Oct. 20-24	12.4	8.9	128	.587	4.7	.23	1.08	4.07	32.8	1.44	1.77	14.2	1.30	72.0	.25	16.2	.22	11.8	93.1	752	112.8	872
5	Oct. 25-29	7.7	16.2	40	.340	4.4	.05	0	2.87	37.0	1.27	1.23	15.6	.60	50.7	.12	7.9	.51	41.4	13.3	169	68.9	902
6	Oct. 31 to Nov. 6	19.6	16.5	88	.663	4.0	.09	1.89	6.06	36.4	2.73	3.75	22.8	1.08	29.6	.14	4.3	2.53	66.1	25.6	148	128.0	757
7	Nov. 14-16	13.5	18.0	73	.594	4.4	.02	1.47	4.90	36.2	1.06	2.15	17.3	.65	27.6	.13	6.7	1.37	65.7	40.3	148	135.5	927
8	Nov. 16-21	15.0	13.7	95	.547	3.6	.17	1.87*	2.72	18.1	1.38	2.72	18.1	1.38	50.8	.03	.2	1.31	48.1	52.6	351	122.0	812
9	Daily Figures	11.7	21.0	44	.336	2.9	.02	4.66	39.8	4.53	38.6	.88	19.4	.15	3.1	3.50	77.5	76.3	65	93.5	797
		15.4	24.4	48	.443	2.9	.02	5.05	32.8	6.82	44.2	1.81	26.5	.63	9.3	4.38	64.2	19.0	123	148.5	964
		9.6	32.7	20	.266	3.1	0	3.18	33.2	4.16	43.4	.87	20.9	.40	9.7	2.89	69.4	8.9	93	53.9	562
		35.6	22.9	120	.856	2.4	0	10.85	30.6	19.20	54.0	6.16	32.0	.96	5.0	12.08	63.0	78.0	219	240.8	628
		12.9	11.7	99	.609	4.7	.11*	1.34*	3.49	26.9	1.91*	2.20	17.8	1.60	70.2	.35	15.4	.34	14.4	71.8	551	130.9	1,012
10	Daily Figures	12.5	8.2	142	.511	4.1	4.58	36.5	2.06	16.4	1.55	73.5	.48	24.9	.03	1.6	84.2	666	114.6	931
		11.8	11.1	96	.502	4.3	3.62	30.9	2.15	18.1	1.05	49.0	.43	18.4	.66	32.6	59.4	505	117.4	1,015
		3.1	14.6	18	.207	6.8	.08*	1.88*	.80	26.3	3.55*	.88	20.0	.42	48.0	.22	24.8	.24	27.2	6.7	218	106.2	3,490
		16.5	20.8	63	.807	4.9	5.38	32.7	2.75	16.7	1.20	43.7	.26	9.6	1.29	46.7	23.1	140	137.8	838
		20.0	15.3	111	.960	4.8	7.07	35.4	3.46	17.3	2.46	71.1	0	0	1.00	28.9	21.2	106	188.0	943
11	Daily Figures	12.0	15.4	66	.616	5.1	3.68	30.8	2.22	18.6	.90	40.3	0	0	1.32	59.7	32.5	272	118.0	988
		25.5	18.1	116	1.188	4.7	8.83	34.6	5.72	22.4	2.52	44.2	.59	10.3	2.61	45.5	61.1	239	183.0	718
		22.9	20.3	90	.867	3.9	5.72	25.0	8.02	35.0	5.99	74.9	2.03	25.1	0	0	100.8	440	166.5	728

* Average.

Case 2, Jacob S.

1	Feb. 27-29	11.6	15.9	62	.672	5.8	.04	1.25	3.35	28.9	1.58	1.53	13.1	.70	45.4	.58	37.8	.26	16.8	51.4	442	99.0	853
2	Feb. 29 to March 4	8.7	21.2	32	.506	5.8	.02	1.13	2.54	29.4	1.42	1.45	16.7	.73	50.3	.02	1.4	.70	48.3	22.0	254	92.6	1,068
3	March 4-7	15.4	14.8	89	.855	5.6	.06	1.66	4.12	26.8	2.22	3.50	23.2	1.79	50.2	.97	27.1	.81	22.7	77.4	504	135.6	883
4	March 7-10	10.8	15.5	59	.685	6.4	.12	1.55	3.22	29.8	1.75	.74	6.8	.45	60.8	0	0	.20	39.2	33.1	307	100.0	1,011
5	March 10-13	9.8	20.9	27	.546	5.6	.05	1.61	3.41	34.7	1.82	.73	7.4	.30	41.1	.07	9.6	.36	49.3	3.4	35	93.3	951
6	March 13-18	11.5	31.4	25	.576	5.0	.04	1.81	4.18	36.4	2.52	1.28	11.1	.42	32.8	0	0	.86	67.2	-3.6	-31	112.2	975
7	March 18-27	9.2	29.1	23	.450	4.9	.03	1.58	3.67	39.7	1.98	.83	9.0	.14	16.7	0	0	.69	83.3	-5.5	-60	73.9	801
		5.2	33.8	10	.264	5.0	.01	.62	2.12	40.5	1.15	.15	2.9	.12	75.9	0	0	.04	24.1	-4.8	-91	119.0	2,275
		8.6	32.4	18	.430	5.0	.03	1.46	3.55	41.3	1.88	1.16	13.5	.27	23.0	.06	5.2	.83	71.8	0	0	104.0	1,213
		46.7	21.6	170	2.519	5.4	.17	4.66	11.25	24.1	6.22	10.84	23.2	7.66	70.7	.14	1.3	3.04	28.0	168.3	360	336.0	719
		28.1	16.9	138	2.454	8.7	.17	1.92	3.86	13.7	2.06	5.04	17.9	4.98	98.9	.06	1.1	0	0	183.6	652	154.0	548
		26.8	13.9	166	2.411	9.0	.25	2.00	4.55	17.0	2.60	5.16	19.3	4.76	92.2	0	0	.40	7.8	245.0	916	177.2	662
		21.4	13.5	137	1.675	7.8	.27	1.62	3.56	16.7	1.90	3.33	15.6	3.05	91.7	.28	8.3	0	0	120.0	562	168.8	790
		29.4	13.3	192	2.233	7.6	.51	2.10	4.89	16.6	2.32	5.97	20.3	5.41	90.6	0	0	.56	9.4	184.5	627	154.4	524
		20.5	13.6	130	1.083	8.2	.38	1.26	3.00	14.6	1.35	3.37	16.4	2.20	65.3	1.09	32.6	.08	2.4	113.8	555	103.5	504

Case 3, Francis D.

1	May 1	20.2	18.4	90	.785	3.9	.10	.88	3.54	17.5	2.34	9.53	47.1	1.46	15.2	.44	4.7	7.63	80.1	40.5	200	162.4	803
2	May 2-4	15.4	17.5	72	.559	3.7	.10	.85	3.34	21.6	1.87	6.31	40.9	1.54	24.4	.05	.7	4.72	74.9	26.6	173	203.0	1,318
3	May 4-19	17.8	13.5	114	.694	3.9	.20	1.49	4.87	27.3	2.73	5.27	29.6	1.01	19.2	.07	1.3	4.19	79.5	29.6	166	131.8	740
4	May 20-24	13.1	13.2	98	.626	4.6	.27	1.30	3.81	27.7	2.18	4.47	32.5	1.22	27.4	0	0	3.25	72.6	23.8	173	124.5	907
		12.1	13.2	70	.605	5.0	.18	.86	2.42	20.1	1.26	4.20	34.8	1.79	42.6	.59	14.1	1.82	43.3	50.3	417	135.0	1,038
		27.3	11.0	220	1.470	5.4	.62	1.98	6.06	22.2	3.25	9.30	33.6	4.34	46.7	3.99	42.9	.97	10.4	139.0	509	320.0	1,173
		17.9	9.3	174	1.013	5.7	.75	1.11	3.57	20.0	1.78	5.42	30.3	2.70	40.8	.90	18.2	1.73	32.0	111.1	623	152.8	1,023
		25.9	11.4	201	1.422	5.5	.46	1.54	4.48	17.3	2.14	6.91	26.7	4.50	65.2	0	0	2.41	34.8	163.0	631	204.0	789

TABLE 5.
BALANCES. AVERAGE DAILY VALUES.
Case 1, Francis H.

Period.	Dates.	Total Nitro- gen Retained, gm.	Nitrogen Retained, per cent. Intake.	Nitrogen Absorbed, per cent.	Nitrogen Absorbed, per cent. Intake	Total Fat Absorbed, gm.	Fat Absorbed, per cent. of Intake.	Chlorid as NaCl Retained, gm.	Phosphates as P ₂ O ₅ Retained, gm.	Total Ash as Oxids Retained, gm.	CaO Retained, gm.	Gain or Loss in Body Weight, gm.	Weight Beg. and End of Period, gm.
1	Sept. 14 to Oct. 3	+1.17	24.5	29.4	83.5	+25.5	78.6	+258	+1.07	+2.04	+1.19	+ 11.7	4.330
2	Oct. 4-14	+ .65	14.4	17.1	84.7	+29.0	86.5	+ .014	+1.12	+ .32	+1.87	+ 4.6	4.553
3	Oct. 16-19	+1.03	15.6	18.1	86.2	0	-.17	+ .51	+ .54	+1.32	+ 25.7	4.683
4	Oct. 20-24	+ .69	12.4	13.9	89.6	+18.2	91.0	-.25	+ .59	+ .36	+ .90	- 23.3	4.781
5	Oct. 25-29	+ .60	10.4	11.1	94.1	+21.8	94.8	-.58	+1.63	+2.36	+1.53	+ 55.3	4.884
6	Oct. 31 to Nov. 6	+3.01	27.0	28.7	94.2	+40.5	91.6	+7.5	+1.28	+3.86	- .33	+ 64.8	4.791
7	Nov. 14-16	+1.99	25.1	27.1	92.7	+41.3	95.0	+98	+1.06	+2.54	+ .76	+ 10.5	4.372
8	Nov. 16-21	+5.62	49.1	51.4	95.3	+29.1	91.5	+388*	+1.027*	+3.97	+ .75*	+ 47.0	4.605
		+2.58	24.7	25.5	96.7	+32.1	87.9	+4.27	+ 1.0	4.994
		+3.28	26.3	27.3	96.5	+32.9	82.8	+4.98	+ 72.0	5.016
		+1.30	11.9	12.3	97.3	+29.6	87.7	+5.35	- 70.0	5.063
		-2.32	84.9	- 3.5	-6.82	-497.0	5.136
9	Dec. 12-16	+1.00	20.2	23.1	87.9	+23.9	91.4	-.05	+ .61	+ .05	- .35*	- 30.3	5.066
	16-20	+1.41	28.2	31.4	89.9	+18.6	90.4	-.07	+ .37	+6.05	+ 50.3	4.569
	20-23	+1.16	23.3	26.0	90.1	+27.8	93.1	-.50	+ .56	+1.22	+ 9.0	5.206
													5.085
													5.085
													5.286
													5.313

* Average.

Case 1, Francis H.—Continued.

10	Dec. 26 to Jan. 1	Daily Figures					96.1	+ 9.72	91.6	+ 3.58*	+ .384*	+ 1.37	— .40*	— 276.0	5.265
		+1.08	21.8	22.7	22.7	22.7	92.1	+25.2	90.2	+ .30	+ 226.0	4.989
		+2.73	26.8	29.2	29.2	29.2	91.8	+22.6	90.3	+1.90	+ 27.0	5.215
		+2.69	23.1	25.2	25.2	25.2	95.3	+21.6	90.6	+4.95	— 33.0	5.242
		+3.48	26.4	27.7	27.7	27.7	90.6	+17.8	75.7	+1.23	+ 109.0	5.209
		+2.51	20.1	22.2	22.2	22.2	53.8	— 4.3	— 5.88	— 697.0	5.318
		— 6.06									4.621

Case 2, Jacob S.

3	March 4 March 4-7	Daily Figures					87.7	+25.1	94.2	+ .22	+ .42	— 1.18	+ .38	+ 92.0	3.968
		+1.89	35.1	40.0	40.0	40.0	92.4	+25.6	94.8	+ .08	+ .83	+ .75	+ .66	+ 14.0	4.151
		+1.62	25.0	27.0	27.0	27.0	87.6	+25.5	87.9	+ .131	+ .268	— .26	+ .04	— 6.0	4.208
4	March 7-10	+1.17	17.1	19.5	19.5	19.5	91.8	+24.2	97.2	+ .437	+ .78	+ 1.20	+ .61	+ 29.0	4.191
5	March 10-13	+1.77	21.3	23.2	23.2	23.2	94.0	+23.6	97.1	+ .128	+ 1.01	+ 1.63	+ .89	+ 16.0	4.278
6	March 13-18	+2.13	23.6	25.1	25.1	25.1	94.2	+15.4	92.3	+ .029	+ 1.08	+ 1.74	+ .78	+ 19.0	4.326
		+1.62	16.4	17.4	17.4	17.4	94.2	+13.8	94.5	— .42	+ .31	+ .86	+ .80	— 75.0	4.423
7	March 18-27	+1.22	15.9	16.9	16.9	16.9	97.8	+15.1	99.0	+ .28	+ 2.17	+ 60.0	4.423
		+3.98	37.7	38.7	38.7	38.7	95.6	+15.9	93.4	+ .14	+ .77	+ 2.34	+ 1.56	— 19.0	4.348
		+1.34	14.0	14.6	14.6	14.6	56.1	+ 3.4	23.6	— .39	— 3.29	— 8.17	— 4.11	— 313.0	4.408
		— 3.20	74.9	+ 11.1	68.6	+ .11	+ .65	+ 1.78	+ .88	— 21.0	4.389
		+1.98	20.5	27.4	27.4	27.4	71.7	+ 9.11	63.8	+ .10	+ .20	+ .67	+ .04	— 35.0	4.076
		— .15	84.0	+ 10.8	76.4	+ .29	+ 1.46	+ 3.28	+ 1.86	+ 21.0	4.055
		+3.66	34.8	41.4	41.4	41.4	72.4	+ 8.9	60.0	— .20	+ .43	+ .43	+ .30	— 46.0	4.020
		+ .15	1.9	2.6	2.6	2.6	70.2	+ 11.4	77.4	— .08	+ .09	+ .49	+ .34	— 127.0	4.041
		— .90									3.995
															3.868

* Average.

Case 3, Francis D.

Period	Date.	Daily Figures												Gain or Loss in Body Weight, gm.	Weight Beg. and End of Period, gm.
1	May 1	Total Nitrogen Re- tained, gm.	Nitrogen Re- tained, per cent, Intake.	Nitrogen Re- tained, per cent, Intake.	Nitrogen Ab- sorbed, per cent, Intake.	Total Fat Absorbed, gm.	Fat Ab- sorbed, per cent, of Intake.	Chlorid as NaCl Retained, gm.	Phosphates as P ₂ O ₅ Retained, gm.	Total Ash as Oxides Re- tained, gm.	CaO Re- tained, gm.			+147.0	4,725
2	May 2-4	+2.96	29.2	31.7	92.2	+28.6	75.2	+768	+1.53	+1.58	+ .80			—	4,872
3	May 4-19	+3.32	29.0	30.5	95.2	+31.7	83.5	+505	+1.74	+3.16	+1.74			—	4,872
4	May 20-24	+3.42	28.5	30.3	94.3	+33.3	86.4	+502	+1.42	+3.10	+1.29			+	4,790
		+3.47	29.5	31.2	94.7	+31.7	87.7	—192	+1.32	+2.68	+1.18			+	5,077
		+6.56	54.9	57.8	94.9	+37.2	89.8	+161	+2.34	+6.07	+2.90			—	5,148
		+2.40	21.1	24.2	87.1	+30.9	76.9	—360	+ .52	+1.04	+ .44			—	5,087
		+2.77	22.6	24.6	91.8	+32.1	85.7	—390	+1.66	+3.51	+2.07			—	5,004
		+2.27	20.5	23.6	87.1	+29.5	81.1	—295	+ .98	+1.38	+1.25			—	4,996
														—	4,793

TABLE 6.
Preliminary Observations on Case 3.

Period.	Dates.	Composition of Food.	Body Weight, gm.	Urine, c.c.	Specific Gravity of Urine.	Indican in Urine.	Intake Chlorids as NaCl, gm.	Chlorids as NaCl in Urine, gm.	Phosphates as P_2O_5 in Urine, gm.
1	January 5-6	Fat 1.5 per cent., carbohydrate 2.2 per cent.	3.862	630	1.009	Faint trace	1.46†	.895	.529
	6-7	Protein 3.4 per cent.	4.076	490	Negative608	.586
	7-8		4.027	505	Faint trace869	.539
2	8-9	Fat 1.6 per cent., carbohydrate 2.3 per cent.	3.957	355*	Faint trace	1.40†	.693	.354
	9-10	Protein 4.0 per cent.	4.102	570	Faint trace775	.405
	10-11		4.090	500	Faint trace770	.406
3	11-12	Fat 1.7 per cent., carbohydrate 2.0 per cent.	4.114	410	1.013	Considerable	1.35†	.872	.416
	12-13	Protein 4.6 per cent.	4.051	390	1.013	Considerable924	.371
	13-14		3.998	370	1.014	Considerable	1.314	.357
4	14-15	Fat 1.7 per cent., carbohydrate 2.1 per cent., protein 5.1 per cent.	3.948	340	Considerable	1.19	.565	.346
	15-16	Fat 1.8 per cent., carbohydrate 2.3 per cent.	3.961	295	1.019	Trace	1.27†	.541	.266
	16-17	Protein 5.5 per cent.	3.944	295	1.015	Faint trace506	.229
6	17-18		3.914	400	1.019	Trace570	.467
	18-19	Fat 1.5 per cent., carbohydrate 2.4 per cent.	4.034	420	1.019	Small trace	1.31†	.422	.449
	19-20	Protein 6.0 per cent.	4.059	280	1.021	Considerable689	.348
7	20-21		4.093	440	1.021	Negative142	.469
	21-22	Synthetic food, 1.8 per cent., carbohydrate 1.8 per cent.	4.026	250*	1.020	Considerable695	.318
	22-23	Protein 5.9 per cent.	4.045	490	1.016	5-10 per cent.	.579†	.450	.406
	23-24	Protein 5.9 per cent.	4.090	480	1.016	20-25 per cent.085	.535
	24-25		3.980	520	1.014	Small trace	0	.540
	25-26		4.026	490	1.018	10-15 per cent.	0	.572
	26-27		3.939

* Loss. † Average.

ON THE KYRINE FRACTION OBTAINED ON PARTIAL HYDROLYSIS OF PROTEINS.*

FIRST COMMUNICATION.

BY P. A. LEVENE AND F. J. BIRCHARD.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

Few facts of general character regarding the structure of the protein molecule have been added since the work of Fischer. The one very important contribution was that of Siegfried, based on his discovery of kyrines. The significance attributed to kyrines consisted in the hypothesis that they constituted fragments of the protein molecule, resembling natural protamines, and hence their discovery substantiated the theory of Kossel that protamines represent the nucleus to which other amino-acids join on in order that a complex molecule of protein may be formed. Whether or not Siegfried has proven Kossel's hypothesis is a problem still awaiting solution, but this investigator undoubtedly has indicated a process by which can be accomplished a partial hydrolysis of protein, leading to the formation of comparatively simple peptides. *A priori*, it seemed logical to expect that if more than one-basic peptides were formed in the process of hydrolysis they would all appear in the general fraction named by Siegfried "kyrine sulphate." Hence it seemed desirable to investigate more closely the composition of the kyrine fraction obtained on partial hydrolysis of protein with the view of a possible discovery in it of more than one peptide.

The expectation was realized inasmuch as the crude kyrine fraction obtained from gelatin was fractionated into two peptides. This conclusion has forced itself on us first as a result of the comparison of the ratio $\frac{\text{Amino N}}{\text{Total N}}$ before and after hydrolysis of the kyrine

* Received for publication, October 3, 1912.

mixture. The arguments can be made more obvious by discussing the details of the analytical data.

Following the directions of Siegfried, a substance was obtained from gelatin, resembling in every way kyrine sulphate, which was further purified by means of silver sulphate and barium hydrate. The substance obtained after this purification had the following properties: Its elementary composition resembled that of Siegfried's kyrine; its phosphotungstic compound crystallized in the same form as described by Siegfried for the same derivative of kyrine; on hydrolysis it gave arginine, lysine, glutaminic acid, glycoll and proline. The presence of the latter was demonstrated by the indirect method. These amino-acids were combined in peptide linking to some extent. The reason for this assumption is the following: If the substance were a mixture of amino-acids the ratio of the amino nitrogen to the total nitrogen should have been 5:9. Since in arginine three nitrogen atoms do not function as primary amino nitrogen, and since the nitrogen of proline must be placed in the same class, in a mixture of the five named amino-acids containing nine nitrogen atoms, five possess the properties of primary amines in their behavior to nitrous acid.

The ratio of amino nitrogen to total nitrogen in our substance was only from 32 to 33 per cent, indicating that some of the primary amino nitrogen atoms had ceased to function as such, presumably through the formation of peptide linkings.

The next task was to ascertain whether or not the five amino-acids were combined in one or in more than one peptide. If the five acids were linked into one peptide this should contain only two nitrogen atoms in the form of primary amino nitrogen, namely, that of the end-acid and the one in the ω -position in the lysine. Thus a pentapeptide composed of the five named amino-acids requires a ratio of amino nitrogen to total nitrogen of 2:9 or of 22 per cent. The substance obtained by us contained 32 per cent of amino nitrogen. Hence it was composed of more than one peptide.

These data, and the data obtained on the nitrogen partition after hydrolysis, led us to the belief that the substance consisted of two basic peptides: one containing lysine and three mono-amino-acids,

and the other arginine and one amino-acid. This assumption was corroborated by the fact that it was possible to separate the mixture into two fractions by means of silver sulphate and barium hydrate. One of these, arginine glutaminic acid peptide, was about 95 per cent pure. The degree of purity was demonstrated by the ultimate analysis of its sulphate, by the ratio of amino nitrogen before and after hydrolysis, by the quantitative estimation of arginine contained in it and by the fact that both arginine and glutaminic acid were obtained from it on hydrolysis. The second peptide has not yet been obtained in the same degree of purity. The nitrogen partition of the pure peptide requires a ratio of 40 per cent of amino nitrogen; instead of which, the substance contained only about 37 per cent. It is hoped that this peptide will also be obtained in a sufficient degree of purity before very long. Work on the separation of the basic peptides from other proteins is at present in progress.

The substance discussed here, with the $\frac{\text{Amino N}}{\text{Total N}}$ ratio of 32 per cent, was not exactly the kvrine sulphate of Siegfried, but was chosen for the reason that it offered a more convenient material for presenting our arguments. The kyrine prepared according to Siegfried by repeating the precipitation with alcohol five times had the ratio $\frac{\text{Amino N}}{\text{Total N}} = 23.6$ per cent. By means of one treatment with silver sulphate and barium hydrate it was separated into two fractions, one of which consisted principally of the dipeptide, the other being a mixture having the ratio $\frac{\text{Amino N}}{\text{Total N}} = 32$ per cent. Hence it is evident that the substance obtained by us according to the directions of Siegfried contained a higher proportion of the dipeptide than the mixture discussed in this communication.

However, it should be borne in mind that only slight changes in the conditions of hydrolysis may bring about considerable variations in the composition of the products obtained through it, and hence it is possible that the substance analyzed by Siegfried had a composition different from that of the substance obtained by us.

EXPERIMENTAL PART.

The original material was prepared according to the method of Siegfried for the preparation of gluto-kyrine¹ and need not be discussed here. It was analyzed as sulphate after drying in vacuum to constant weight at the temperature of boiling chloroform for one day and at the temperature of boiling alcohol for seven days, and had the following composition.

Ash, 1.8 per cent.

Substance, 0.2250 gram; CO₂, 0.2948 gram; H₂O, 0.1248 gram.

Substance: 0.1538 gram required 17.65 cc. of N/10 acid.

Substance: 0.2618 gram gave 0.2032 gram BaSO₄.

	Substance here described.	Kyrine sulphate of Siegfried.
C	35.70 per cent.	31.86 per cent.
H	6.22 per cent.	5.61 per cent.
N	16.06 per cent.	15.98 per cent.
S	10.65 per cent.	10.13 per cent.

Amino Nitrogen Ratio in the Original Kyrine Sulphate.

The ratio of amino nitrogen to total nitrogen was determined according to the method of Van Slyke. One gram of substance was dissolved in 50 cc. of water and the total nitrogen determined according to Kjeldahl and the amino nitrogen according to Van Slyke.

Total N: 10 cc. solution neutralized 21.8 cc. N/10 acid. N = 30.52 mgm.

Amino N: 10 cc. solution gave 12.7 cc. gas at 19°, 754 mm. N = 7.20 mgm.

Amino N

Total N 23.61 per cent.

The substance was ammonia-free. 0.5 gram substance distilled with Ca(OH)₂ and methyl alcohol under diminished pressure neutralized 0.0 cc. N/10 acid.

Purification of the Kyrine Sulphate.

The kyrine prepared according to Siegfried's directions was further purified by treatment with silver sulphate and barium hydrate as in Kossel's method for the separation of arginine from the hydrolytic mixture. The kyrine sulphate was dissolved in a few cubic centimeters of water and a hot solution of silver sulphate added,

¹ Bericht der math.-physik. Klasse der königl. sächsischen Gesellschaft der Wissenschaften zu Leipzig, March 2, 1903.

care being taken to constantly cool the resulting liquid by surrounding it with ice. The addition of silver sulphate was continued until a drop of the mixture added to a solution of barium hydrate on a watch glass produced a brown coloration. After cooling, finely powdered barium hydrate was slowly added to complete saturation, the liquid being constantly stirred mechanically. The precipitate was filtered off with the aid of suction, the whole being kept in a refrigerator at approximately 0° . The precipitate and the filtrate were then slightly acidified with sulphuric acid and decomposed with hydrogen sulphide. The resulting solutions were finally freed from sulphuric acid, concentrated to a small volume under diminished pressure, the sulphates formed by the addition of sulphuric acid and each precipitated in a large excess of absolute alcohol. After standing for some time in the refrigerator at 0° the peptides were filtered off with the aid of suction, washed with absolute alcohol and ether and dried in a desiccator over sulphuric acid.

Nitrogen Partition in the "Purified Kyrine" before Hydrolysis.

0.75 gram substance was hydrolyzed with 20 per cent hydrochloric acid for twenty hours. The excess of hydrochloric acid was then distilled off under diminished pressure, the residue taken up in water, exactly neutralized with sodium hydrate and finally brought to a volume of 50 cc. In a duplicate experiment 1.4 grams of substance were treated in the same manner.

(1) Total N: 10 cc. solution required 15.8 cc. N/10 acid.	N = 22.12 mgm.
Amino N: 10 cc. solution gave 12.8 cc. gas, 19° , 760 mm.	N = 7.28 mgm.
Amino N	
Total N	32.9 per cent.
(2) Total N: 10 cc. solution required 38.7 cc. N/10 acid.	N = 54.21 mgm.
Amino N: 10 cc. solution gave 31.5 cc. gas, 25° , 758 mm.	N = 17.46 mgm.
Amino N	
Total N	32.2 per cent.

The Ratio of Amino Nitrogen to Total Nitrogen after Hydrolysis.

0.5 gram of the substance was hydrolyzed with 30 cc. of a 20 per cent solution of hydrochloric acid for twenty hours over flame with return condenser. The excess of hydrochloric acid was removed by distillation, the residue taken up in water, neutralized with sodium hydrate and brought to a volume of 50 cc.

Total N: 10 cc. solution required 12.89 cc. N/10 acid.	N = 18.04 mgm.
Amino N: 10 cc. solution gave 20.4 cc. gas, 19°, 760 mm.	N = 11.69 mgm.
Amino N	
Total N	64.7 per cent.

Nitrogen Partition in Phosphotungstic Acid Precipitate and Filtrate after Hydrolysis.

One gram of substance was hydrolyzed with a 20 per cent solution of hydrochloric acid for twenty hours over flame with return condenser. After removing the hydrochloric acid by repeated concentration under diminished pressure the residue was taken up with 10 per cent sulphuric acid and a 10 per cent solution of phosphotungstic acid added. The solution and precipitate so formed were then heated on the water bath until the precipitate was nearly all dissolved and then allowed to stand until the next day. The nitrogen in the precipitate and filtrate was determined according to Kjeldahl.

The precipitate required 44.60 cc. of N/10 acid and the filtrate 25.92 cc. of N/10 acid. Hence the ratio of nitrogen in the phosphotungstic precipitate to the total nitrogen = $44.60/70.52 = 63.25$ per cent.

Amino Nitrogen Ratio in the Phosphotungstic Acid Precipitate and Filtrate.

In a second portion the ratio of amino nitrogen to total nitrogen in the phosphotungstic acid precipitate and filtrate after acid hydrolysis was determined. The precipitate was brought into solution with the aid of dilute alkali and brought to a volume of 30 cc.

Total N: 10 cc. solution gave 9.19 cc. N/10 acid.	N = 38.59 mgm. in toto.
Amino N: 10 cc. solution gave 12 cc. gas, 20°, 760 mm.	N = 20.52 mgm. in toto.
Amino N	
Total N	53.1 per cent.

The filtrate was concentrated to a volume of 50 cc.

Total N: 35 cc. solution required 15 cc. N/10 acid.	N = 30.0 mgm. in toto.
Amino N: 10 cc. solution gave 7.9 cc. gas, 19°, 760 mm.	N = 22.53 mgm. in toto.
Amino N	
Total N	75.1 per cent.

Hydrolysis of the Kyrine Fraction Aiming to Isolate the Individual Components.

About 45 grams of the sulphate were hydrolyzed with a 20 per cent solution of hydrochloric acid for twenty-four hours. The solution was then treated with phosphotungstic acid in the manner before described, the precipitate was washed chlorine-free with 5 per cent sulphuric acid, and the phosphotungstic and sulphuric acids were removed quantitatively with barium hydrate. The resulting liquid was concentrated to a small volume and to a portion an alcoholic solution of picrolonic acid was added and allowed to stand over night. The precipitate so obtained was recrystallized out of water and analyzed.

Substance, 0.1600 gram; CO_2 , 0.2541; H_2O , 0.0706.

	Calculated for $\text{C}_9\text{H}_{14}\text{N}_4\text{O}_5 \cdot \text{C}_{10}\text{H}_8\text{N}_4\text{O}_6$	Found.
C	43.82	43.23
H	5.05	4.90

Lysine was isolated from the phosphotungstic acid precipitate according to Kossel's method and obtained as the picrate. It was identified by determining the amino nitrogen.

Substance, 0.0930 gram; gas, 12.8 cc., 20° , 764 mm.

	Calculated for $\text{C}_6\text{H}_{14}\text{N}_2\text{O}_5 \cdot \text{C}_6\text{H}_5(\text{NO}_2)_3\text{OH}$	Found.
Amino N	7.48	7.86

Glutaminic acid was separated from the phosphotungstic acid filtrate as hydrochloride after removal of the sulphuric and phosphotungstic acids quantitatively by barium hydrate.

0.1500 gram substance gave 0.1153 gram silver chloride.

	Calculated for $\text{C}_6\text{H}_5\text{NO}_4 \cdot \text{HCl}$	Found.
Cl	19.31	19.01

The excess of hydrochloric acid was removed by concentration under diminished pressure and the remainder with silver sulphate. The silver was removed with hydrogen sulphide and the sulphuric acid with barium hydrate and the volume brought to 200 cc.

Total N: 5 cc. required 17.85 cc. N/10 acid. N = 24.94 mgm.

Amino N: 5 cc. gave 32.2 cc. gas, 20° , 760 mm. N = 18.30 mgm.

The ratio of amino nitrogen to total nitrogen in the phosphotungstic acid filtrate = 73.3 per cent or 26.7 per cent of the nitrogen is non-amino nitrogen. This indicates the presence of proline or oxyproline.

The copper salts were formed from the remainder of the filtrate and extracted with hot alcohol. On standing a large portion crystallized out. The portion soluble in hot and cold alcohol appeared on analysis to be largely the copper salt of proline or oxyproline. It was dissolved in 20 cc. of water.

Total N: 10 cc. required 20.43 cc. N/10 acid. N = 28.60 mgm.

Amino N: 10 cc. gave 17 cc. gas, 18°, 764 mm. N = 9.81 mgm.

$\frac{\text{Amino N}}{\text{Total N}} = 34.3$ or 65.7 per cent of the nitrogen of the phosphotungstic acid filtrate is non-amino.

The nitrogen content of the crystals which separated from the hot alcohol did not correspond to any of the ordinary amino-acids.

0.0964 gram substance required 7.3 cc. N/10 acid. N = 10.22 mgm.

Total N 10.60 per cent.

0.0991 gram substance gave 18.4 cc. gas, 20°, 764 mm. N = 10.53 mgm.

Amino N 10.63 per cent.

The analysis of the copper salt of the portion insoluble in hot and cold alcohol indicated a mixture, probably of alanine and glycocoll.

0.2154 gram substance gave 0.2146 gram CO₂; 0.0910 gram H₂O.

	Calculated for (C ₂ H ₄ NO ₂) ₂ Cu.	Calculated for (C ₃ H ₅ NO ₂) ₂ Cu.	Found.
C	25.00	30.00	27.28
H	4.16	5.00	4.60

The results of the hydrolysis show that arginine, lysine and glutamic acid are certainly present and probably also proline. Glycocoll may also be present and perhaps alanine.

Preparation of the Dipeptide.

The precipitate obtained on treatment of the solution of the original kyrine with silver sulphate and barium hydrate contained

the dipeptide. It was freed from silver and barium in the manner described earlier in the paper and transformed into the sulphate in the same manner as the original kyrine. The dipeptide nature of the substance was based on the $\frac{\text{Amino N}}{\text{Total N}}$ ratio before and after hydrolysis, on the fact that on hydrolysis it yielded arginine and glutaminic acid and on its elementary composition. A dipeptide composed of arginine and glutaminic acid should contain only one free primary amino group out of five nitrogen atoms in the molecule, or the $\frac{\text{Amino N}}{\text{Total N}}$ is calculated at 20 per cent. The values found on analysis of the substance only slightly exceeded that value.

The ratio of the nitrogen in the form of amino-acids to the basic nitrogen is calculated also at 20 per cent, and the analyzed body showed the value of the nitrogen of amino-acids to represent 21.1 per cent of the total nitrogen.

Furthermore, on hydrolysis of the dipeptide, free arginine and free glutaminic acid are formed, hence the mixture of the two contains twice the number of amino groups as compared with the original number. The value found on hydrolysis of the analyzed body only slightly exceeded the theoretical value.

Finally the arginine determination made directly on the peptide gave results deviating only slightly from the value theoretically calculated.

Elementary Analysis of the Dipeptide.

The dipeptide was analyzed as sulphate. It was dried under diminished pressure at room temperature over sulphuric acid for several days and then at the temperature of boiling chloroform for two days and at that of boiling carbon tetrachloride for five days.

Substance, 0.2407 gram; CO₂, 0.2943; H₂O, 0.1242.

Substance, 0.1808 gram; CO₂, 0.2160; H₂O, 0.0916.

0.3209 gram substance required 40.1 cc. N/10 acid = 56.14 mgm. N.

0.4033 gram substance gave 0.2712 gram BaSO₄.

0.1952 gram substance gave 0.1276 gram BaSO₄.

	Calculated for (C ₁₁ H ₂₁ N ₅ O ₈)H ₂ SO ₄	Found.
C	32.90	32.97
H	5.73	5.72
N	17.40	17.48
S	8.20	9.11

*Hydrolysis of the Dipeptide Indicating the Nitrogen Partition
Before and After Hydrolysis.*

Two solutions of the sulphate were prepared, the first containing approximately 0.5 gram of the sulphate in 25 cc. and the second, 1.0 gram in 25 cc. These solutions were used for the determination of the amino nitrogen before hydrolysis. A third solution containing approximately 2.5 grams of the sulphate was hydrolyzed with hydrochloric acid in the usual manner and the nitrogen partition in the phosphotungstic acid precipitate and filtrate determined in the manner described for the "purified kyrine" fraction. The ratio of amino nitrogen to total nitrogen was also determined in the same manner.

Before Hydrolysis.

- (a) Total N: 10 cc. solution required 17 cc. N/10 acid. N = 23.80 mgm.
 Amino N: 10 cc. solution gave 8.7 cc. gas, 20°, 760 mm. N = 4.96 mgm.
 Amino N
 Total N 20.8 per cent.
- (b) Total N: 5 cc. solution required 21.4 cc. N/10 acid. N = 29.96 mgm.
 Amino N: 10 cc. solution gave 22.5 cc. gas, 30°, 758 mm. N = 12.23 mgm.
 Amino N
 Total N 20.4 per cent.

After Hydrolysis.

(1) Nitrogen partition in phosphotungstic acid precipitate and filtrate.

Total volume of the solution, 50 cc. 5 cc. solution required 21.78 cc. N/10 acid. Total N = 304.92 mgm.

Total N in filtrate from phosphotungstic acid precipitate: Total volume of solution, 100 cc. 25 cc. solution required 11.55 cc. N/10 acid. N = 16.17 mgm. Total N = 64.68 mgm.

The ratio of the nitrogen in the phosphotungstic acid filtrate to the total nitrogen = 21.1 or the ratio of the nitrogen in the phosphotungstic acid precipitate to the total nitrogen = 78.9 per cent.

Allowing for the correction of the solubility of arginine phosphotungstate, one finds the nitrogen value of the phosphotungstic precipitate to constitute 79.7 per cent. of the total nitrogen.

(2) *The ratio of amino nitrogen to total nitrogen.* This determination was made on a second portion hydrolyzed as previously described.

Total N: 10 cc. solution required 21.90 cc. N/10 acid. N = 30.55 mgm.

Amino N: 10 cc. solution gave 24 cc. gas, 20°, 760 mm. N = 13.68 mgm.

Amino N	
Total N	44.4 per cent.

Hydrolysis of the Dipeptide.

100 cc. of an aqueous solution of the dipeptide containing 1.22 grams of nitrogen were concentrated to 20 cc. and 2 grams of sulphuric acid added. This was hydrolyzed by heating for five hours in a sealed tube. Water was added so that the solution finally contained 5 per cent sulphuric acid and then was precipitated with phosphotungstic acid. After standing for two days in the refrigerator, the precipitate was filtered off in the cold and washed with 5 per cent sulphuric acid. The phosphotungstic acid was removed from the filtrate by shaking out with ether, the ether distilled off, and the sulphuric acid removed quantitatively with barium hydrate. The resulting solution was concentrated under diminished pressure to a small volume and alcohol added, when a portion crystallized out. This crystallized portion was dissolved in water and converted into the picrolonate and analyzed.

Substance, 0.0998 gram; CO₂, 0.1604 gram; H₂O, 0.0338 gram.

	Calculated for C ₈ H ₉ NO ₄ · C ₁₀ H ₉ N ₄ O ₆	Found.
C	43.75	43.8
H	4.16	3.80

The mother liquors were also converted into the picrolonate. These analyzed for a mixture of an inorganic picrolonate and impure glutaminic acid.

Ash-free substance, 0.1038 gram; CO₂, 0.1702 gram; H₂O, 0.0274 gram; Ash, 0.0022 gram.

C	44.70 per cent.
H	2.95 per cent.
Ash	2.20 per cent.

The phosphotungstic acid precipitate was suspended in water, decomposed with barium hydrate and the acid removed quantitatively. The resulting solution was concentrated under diminished pressure and picrolonic acid added. The resulting crystals were filtered off, washed, dried and analyzed.

Substance, 0.1196 gram; CO₂, 0.1804 gram; H₂O, 0.0420 gram.

	Calculated for C ₉ H ₁₄ N ₄ O ₅ (C ₁₀ H ₈ N ₄ O ₅) ₂ .	Found.
C	44.40	45.05
H	4.27	4.30

Arginine Content of the Dipeptide.

The arginine was determined directly on the peptide without preceding hydrolysis. 10 cc. of an aqueous solution of the substance were boiled in a Kjeldahl flask with 20 cc. water and 15 grams of potassium hydrate in the manner described by Van Slyke² for arginine determination. At the end of ten hours water was added to the flask and the distillation continued for another hour. Of the original aqueous solution of the dipeptide 10 cc. were used for the arginine determination and 5 cc. for the total nitrogen.

Total N: 5 cc. solution required 35 cc. N/10 acid. N = 49 mgm.

Arginine N: 10 cc. solution boiled with 20 cc. water and 15 grams.

KOH neutralized 26.75 cc. N/10 acid = 37.45 mgm. N. Total arginine N = 74.90 mgm.

	Calculated.	Found.
Total Arginine N	80.0	76.5 per cent.

Amino Nitrogen Content in the Mother Liquor from the Dipeptide.

On the basis of the assumption made in the present communication, the filtrate from the silver and barium precipitate should contain a peptide composed of all the other amino-acids recognized among the cleavage products of the original kyrine, except arginine and glutaminic acid. The polypeptide formed in this manner should contain two amino groups and three non-amino nitrogen atoms in the molecule or $\frac{\text{Amino N}}{\text{Total N}}$ ratio = 40 per cent. We have not yet succeeded in obtaining a solution of the pure tetrapeptide; however, after five repeated treatments with silver sulphate and barium hydrate the $\frac{\text{Amino N}}{\text{Total N}}$ ratio of the solution rose from 23 per cent to 36 per cent, thus indicating that with more patience and with more material one might obtain the pure tetrapeptide.

Total N: 5 cc. solution required 16.5 cc. N/10 acid. N = 23.10 mgm.

Amino N: 10 cc. solution gave 29.0 cc. gas, 21°, 772 mm. N = 8.34 mgm.

Amino N
Total N 36.1 per cent.

² This *Journal*, x, p. 26, 1911.

PERMANENT INTUBATION OF THE THORACIC AORTA.*

By ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

In a previous article¹ it was shown that a segment of vein could be grafted successfully on the thoracic aorta. While this procedure might prove to be the ideal treatment of aneurysms on peripheral arteries, the extirpation of the aneurysmal sac and the transplantation of a vascular segment upon the thoracic aorta would be complicated and dangerous. I have attempted, therefore, to determine the proper technique for intubating the aorta, as this operation is simpler and may prove more satisfactory than grafting.

The idea of introducing a tube into the lumen of a vessel is not original. In fact paraffined glass tubes are used in all physiological laboratories for securing an artificial circulation of blood, and the same method has been applied successfully by Brewer to the transfusion of blood in human beings. Some years ago Abbe tried to effect a permanent anastomosis between the cut ends of an artery by means of a glass tube. He presented at the Academy of Medicine of New York a cat in which he had cut the abdominal aorta and had united the ends of the vessel by a glass tube. In other cases coagulation of the blood took place after a short time. It appeared therefore that even if a foreign body could be put temporarily in a vessel without accident, thrombosis would occur sooner or later.

Nevertheless, it was conceivable that thrombosis was preventable, and that the conditions under which a foreign body could be used for repairing a vessel might be ascertained. I extirpated, therefore, part of the anterior wall of the abdominal aorta of a dog, and replaced it by a piece of rubber covered with vaselin. Fifteen months after the operation, the circulation was still normal. The

* Received for publication, April 4, 1912.

¹ Carrel, *Jour. Exper. Med.*, 1912, xv, 389.

vessel wall was intact inside and outside of the rubber. It was found also that the circulation could take place for six days through a glass tube lying free in the abdominal aorta. Thrombosis occurred subsequently owing to displacement of the tube. Encouraged by these results, I operated upon a number of dogs with the purpose of learning the precautions that must be taken in order to make the intubation successful. In all instances the operations were performed on the thoracic aorta of medium-sized dogs. The thoracic cavity was opened under ether anesthesia by the method of Meltzer and Auer, and a glass or metal tube was inserted into the lumen of the descending aorta.

The animal was etherized and the hair on the left side of the thorax was removed by sodium sulphid. The skin was washed with alcohol and covered with 10 per cent. tincture of iodine. Then a catheter was introduced into the trachea and connected with a Meltzer and Auer apparatus which consisted of a foot bellows, an ether bottle, and a small manometer. Next the thoracic cavity was opened by an incision passing through either the eighth or the fourth intercostal space. As soon as the pleura was opened, silk compresses sterilized in vaselin were introduced in order to protect the left lung, and to wall off the pleural cavity. Then a retractor of Gosset was placed between the ribs and opened as much as was necessary. Another silk compress was introduced in the cavity in order to circumscribe completely the operating field. Since sponging irritates the pleura, it is very important that no blood should escape into the cavity.

The pleura was cut along the portion of the aorta that had to be intubated, but the aorta itself was not dissected, and its collateral branches were not ligated. Two ligatures of soft but heavy silk thread were passed around the aorta approximately at the points where the ends of the tube were to be fixed. A few centimeters above and below these two ligatures, two elastic forceps were kept ready for clamping the vessel. The elastic blades of the forceps were applied directly to the arterial wall, no rubber being interposed.

The wall of the aorta was then cut longitudinally. The edges of the incision were caught with two forceps and the tube was introduced into the lumen of the aorta. The tubes were made of glass

or aluminum. They were generally nine or ten millimeters in diameter and forty-five millimeters long. As the dogs were of various sizes, the tubes either distended the vessels, fitted them exactly, or were smaller than the caliber of the aortas. The tubes were straight, but their edges were slightly everted. The glass tubes were crude, their edges being often irregular and sharp. Before being used, the tubes were paraffined. As soon as the tube was inserted into the lumen of the vessel, the silk threads that had been put around the aorta previously, were tied. The ligature should be tight enough to prevent the tube from slipping, but not tight enough to cut the wall of the artery. This wall is easily injured.

The clamps were removed and the circulation was reestablished through the intubated segment. Generally there was no hemorrhage from the incision through the aortic wall, and in some cases the opening was not closed. In others it was sutured with fine silk. It is safer to close the opening carefully even if there be no bleeding. The pleura was then sutured along the aorta. The silk compresses were removed and the ribs were approximated by two silk ligatures. Before closing the thoracic cavity, slight compression was made on the trachea in order that the lungs might regain their normal size. The muscles and then the skin were united by continuous sutures.

Ten experiments were made. In one the introduction of a tube into a comparatively small aorta resulted in tearing the wall, and the animal was killed immediately by chloroform. The nine other cases will be briefly described.

Experiment 1.—Medium-sized bull terrier, No. 729. On January 18, 1912, 10:16 A. M., a transverse thoracotomy was made in the tenth intercostal space, under ether anesthesia by the Meltzer and Auer method; the lower part of the thoracic aorta was dissected. At 10:50 temporary hemostasis was caused by the two pairs of elastic jawed forceps. The aorta was incised and the paraffined glass tube 45 mm. in length and 9 mm. in diameter was introduced. The tube was fixed by an upper ligature of rubber and by a lower ligature of silk, both being very tight. The circulation was reestablished at 10:56. The incision in the arterial wall was not sutured, and the thoracic cavity was closed at 11:18. After the operation the femoral pulse was normal and the animal remained in excellent condition until January 26, when it appeared sick and no pulsation was felt in the right femoral artery. On January 27 the animal was again in good condition, but on January 29, at 10 A. M. it died suddenly.

Autopsy.—The thoracic cavity was filled with blood. The ligatures, which had been too tight, had cut the arterial wall and a sudden hemorrhage resulted.

The tube was in the aorta. No paraffin or fibrin could be seen on its wall. But on the wall of the aorta near the upper end of the tube, there was a slight laceration covered by a thin deposit of fibrin. There was no clot in the iliac arteries.

Experiment 2.—Black and white female dog, No. 769. On January 26, 1912, at 10:20 A. M., a transverse thoracotomy was made in the eighth intercostal space, under ether anesthesia by the Meltzer-Auer method. At 10:28 temporary hemostasis of the middle part of the descending aorta was caused. This was followed by a longitudinal incision of the vessel and the introduction of a paraffined glass tube 45 mm. in length and 9 mm. in diameter. The tube was fixed at each end by a ligature of heavy surgical silk, but the aortic incision was not sutured. The circulation was reestablished at 10:36, and at 10:55 the operation was ended. The animal remained normal until February 5, but on February 5, at 4 P. M. the pulsations of the femoral arteries disappeared, and the dog died shortly afterwards.

Autopsy.—There was no hemorrhage in the thoracic cavity, but a large clot was found adherent to the non-sutured incision of the aortic wall. Longitudinal incision of the aorta revealed the fact that the threads had not cut the arterial wall. The upper end of the tube, which was too sharp, had caused a slight laceration of the vessel wall. The tube was lined with fibrin, except in its lower part. The upper part of the aorta was open, but the lower part was completely obliterated by a thrombus. This had been produced by the fibrin in the lower end of the tube becoming loose and being swept by the blood stream into the lumen of the aorta.

Experiment 3.—Young, brown female dog, No. 798. On January 30, 1912, at 10:21 a thoracotomy was made through the eighth intercostal space under ether anesthesia by the Meltzer-Auer method. At 10:35 temporary hemostasis was caused in the middle part of the descending aorta. The wall was incised, and a paraffined glass tube was introduced. The tube was 45 mm. long and 9 mm. wide. Its caliber proved to be smaller than that of the aorta, and the edge of its upper end was slightly everted. A ligature of heavy silk was put around the aorta at the level of the upper end of the tube, but the lower end was not ligated. The tube fitted the aorta exactly, and the incision was not sutured. At 10:38 the circulation was reestablished, and at 11:05 the thoracic cavity was closed and the operation ended. The animal remained normal.

Experiment 4.—Young, white female dog. On February 6, 1912, at 10:31 under ether anesthesia by the Meltzer-Auer method, a thoracotomy was made through the eighth intercostal space on the left side of the animal. At 10:47 a thin-walled paraffined glass tube 40 mm. long and 9 mm. wide was introduced into the descending aorta by the usual procedure. The tube was fixed by two heavy ligatures which were tied loosely. The circulation was reestablished at 10:52, and at 11:10 the operation was ended. The animal remained normal for eight days, but died suddenly on February 14.

Autopsy.—The thoracic cavity was filled with blood, for the glass tube had broken in the middle, and hemorrhage had taken place through the aortic incision. There was no clot in the tube or in the aorta, and no paraffin or fibrin was seen on the glass wall. There was no ulceration of the aorta at the ends of the tube. In fact the aortic wall was in excellent condition.

Experiment 5.—White and brown female dog, No. 893. On February 9, 1912, at 10:22 A. M., a left thoracotomy was made through the eighth intercostal space under ether anesthesia by the Meltzer-Auer method. At 10:36 A. M. during temporary hemostasis a paraffined glass tube was introduced, and was fixed by two heavy silk ligatures, although the tube seemed too narrow for the aorta. The circulation was reestablished at 10:40. The aortic incision was then sutured. At 10:49 the thoracic cavity was closed, and at 11:02 the operation was ended. The animal remained in good health for twenty-three days, but on April 4, while the dog was running, its posterior limbs became suddenly paralyzed. After about three hours, it was again in normal condition. On April 7, 1912, it was found dead in its cage.

Autopsy.—The aorta was obliterated just below the lower end of the tube by a fibrinous clot. The thrombus originated apparently from a laceration produced in the aortic wall by the lower end of the tube, which was very sharp. The upper end had caused no laceration. The lumen of the tube was entirely free.

Experiment 6.—Black and white, long haired, male dog, No. 920. On February 13, 1912, at 10:25 A. M., under ether anesthesia by the Meltzer-Auer method, the thoracic cavity was opened and the descending aorta was prepared in the usual way. At 10:36 during temporary hemostasis, a paraffined aluminum tube 45 mm. in length by 10 mm. in diameter was introduced into the descending aorta. The tube was fixed by heavy silk ligatures but not very tightly. The caliber of the tube was markedly smaller than that of the aorta. The aortic incision was then sutured. The circulation was reestablished at 10:39, and the thoracic cavity was closed and the operation ended at 11:02. The dog remained in excellent condition. On March 25, the animal was apparently in good health. On March 26 it died rather suddenly.

Autopsy.—The pleural cavity was distended by a clear serosanguineous fluid. The intubated portion of the aorta was surrounded by a large tumor which was composed of granulation tissue. The tube was corroded and partially occluded by a clot of fibrin.

Experiment 7.—Brown female spaniel, No. 940. On February 16, 1912, at 10:13 A. M., under ether anesthesia by the Meltzer-Auer method, a thoracotomy was made on the left side. At 10:29, during temporary hemostasis in the descending aorta, the wall was incised and a paraffined aluminum tube was introduced. The aorta was small and the tube too large for it. At 10:31 the circulation was reestablished. The tube was fixed by two silk ligatures. On account of the distension of the aorta by the tube, the incision could not be sutured. The thoracic cavity was closed and the operation completed at 11 A. M. The animal remained normal for nine days, but on the tenth day (February 26) it died suddenly.

Autopsy.—There was no hemorrhage in the pleura. The tube, however, was occluded by a clot. The deposit of fibrin seemed to have started from a laceration of the wall at the level of the upper end of the tube. The laceration was apparently due in some measure to pressure exerted upon the wall, by the edge of the tube. The tube was corroded and the aortic wall and the surrounding connective tissue were very much thickened.

Experiment 8.—Black and white female dog, No. 992. On February 27, 1912,

at 10:06 A. M., under ether anesthesia by the Meltzer-Auer method, a left thoracotomy was made through the fourth intercostal space. The left part of the arch of the aorta was dissected and at 10:36 the aorta was clamped near the left innominate artery. Into the vessel a straight paraffined glass tube was then introduced. The tube was fixed by two silk ligatures, and at 10:38 the circulation was reestablished and the aortic incision sutured. The pleura was also sutured around the vessel, the operation being finished at 11:12. The animal remained in excellent condition for three days, but on the morning of March 2, it was found dead in its cage.

Autopsy.—The tube was found to be occluded by a clot. This tube was straight and its sharp edges had lacerated the aortic wall. The thrombus appeared to have started in the lacerated region.

Experiment 9.—Black and white female dog, No. 1017. On March 1, 1912, at 10:41, under ether anesthesia by the Meltzer-Auer method, a left thoracotomy was made through the fourth intercostal space. This was followed by dissection of the arch of the aorta, of the first part of the innominate artery, and of the upper part of the descending aorta. At 10:58, during temporary hemostasis, the aortic wall was incised and a curved paraffined glass tube 3 mm. in length and 10 mm. in diameter was introduced into the lumen of the vessel and fixed by two silk ligatures. The circulation was reestablished at 11 A. M. and the incision in the aorta was sutured. The thoracic cavity was then closed and the operation ended at 11:25. The dog remained in excellent condition for five days but died suddenly on March 7, 1912.

Autopsy.—The upper end of the tube was too sharp and had lacerated the wall. To this lacerated surface a clot that completely occluded the tube was adherent.

Thoracotomy, under ether anesthesia by the Meltzer-Auer method, and handling of the thoracic aorta are not dangerous. No post-operative pleuritis or other complication was observed, and during the first week following the operation, nearly all the animals remained in excellent health.

One animal (experiment 3) is living and normal at the beginning of the fourth month after the operation (April 3).² In this animal a glass tube of relatively small caliber with smooth edges was introduced into the descending aorta, and fixed there by a single ligature of coarse silk thread.

In two cases (experiments 1 and 4), the animals died of hemorrhage. In experiment 4 a thin-walled glass tube had been used. Eight days after the operation, the animal was apparently in excellent health, but died suddenly on this day. The pleural cavity was

² On May 2 the animal became suddenly paralyzed and was chloroformed. The autopsy showed that the upper edge of the tube had ulcerated the posterior part of the aortic wall, and that the tube was occluded by a fresh clot.

full of blood and the tube was found broken in the middle. The lumen was free from paraffin and fibrin. There was no ulceration of the aortic wall. In experiment 1 the tube had been fixed by two very tight ligatures which, after eleven days, cut the arterial wall, causing a sudden hemorrhage and the death of the animal. The use of a metallic tube and of ligatures properly tied would have prevented these accidents.

In experiment 6 the animal remained in excellent health for more than fifty days, but then became slightly ill and died rather suddenly, fifty-five days after the operation. Both pleural cavities were filled with a clear hemorrhagic fluid. Around the intubated portion of the aorta was a large tumor adherent to the posterior thoracic wall. This tumor was composed of granulation tissue in the center of which was found the aluminum tube. The tube was corroded and partially occluded by a deposit of fibrin. In experiment 7 an aluminum tube had also been used. The animal died rather suddenly twelve days after the operation. The tube was corroded. Its lumen was obliterated by a thrombus, and the intubated part of the aorta was very much thickened. In both cases the complications could probably have been prevented by the use of tubes made of gold instead of aluminum. In four other cases occlusion of the aorta or of the tube occurred. In three animals (experiments 2, 8, and 9) the deposit of fibrin was due to a laceration of the wall produced by the upper end of the tube. The tubes were too large and had distended the aorta. It is probable that tubes small enough to have produced no distension of the aortic wall would have caused no laceration. In the fourth animal in which an occluded aorta was found (experiment 5), a glass tube with sharp edges had been introduced into the descending aorta. This animal remained in excellent condition for fifty-eight days but then died rather suddenly. The aorta was occluded just below the lower end of the tube by a fibrinous clot that was adherent to a laceration of the wall produced by the edge of the tube. No laceration was caused by the upper end, and the lumen of the tube was entirely free from thrombus. This observation showed that blood could circulate for almost two months through a glass tube without the development of a thrombus. The laceration caused by the sharp edge seemed to be responsible for the obliteration of the vessel.

These experiments have shown that under certain conditions aortic blood can flow through a glass tube for more than three months without the occurrence of an obliterative thrombus. If the aortic wall was lacerated, a deposit of fibrin took place, and caused a partial or complete occlusion of the tube or of the vessel. The success or failure of the intubation depended upon the presence or absence of laceration of the vascular wall. It is probable that the use of a tube of proper caliber, form, and composition, for instance, a smooth edged, gold tube of relatively small caliber, would be followed by better results. It is possible also to line the tube with a vein in order to prevent more surely the occurrence of a thrombus. Further experiments will show whether complications can be completely eliminated, and whether the operation can be made safe enough to be used on human beings in case of aneurysms of the aorta.

PURE CULTURES OF CELLS.*

By ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

The cells surrounding a fragment of tissue growing *in vitro* nearly always belong to several different kinds of tissues. It is important to isolate and cultivate a pure strain of cells of a determined type. I have attempted, therefore, to develop a technique by which pure cultures of cells might be obtained.

In its essentials the technique consists in isolating the cells of the selected type and in propagating them by repeated passages from medium to medium.

During the first few days of the growth of a culture of tissues, it was generally impossible to isolate cells belonging to a definite type. When the culture had undergone several passages, however, the cells grew more actively and spread over a larger area of the medium. Often a large group of cells belonging morphologically to one type could be seen growing in a thin layer. By careful microscopical examination it could be ascertained that no cells of a different type were present.

Then the part of the plasmatic jelly which contained the selected cells was isolated by cutting it away from the remaining part of the culture with a sharp cataract knife. The little film containing the cells was washed for one or two minutes in Ringer's solution and was then placed in a new medium. The new medium was composed of two parts of hypotonic plasma and one part of embryonic extract. A second microscopical examination showed that all the cells placed in this medium belonged to the type selected.

After the cells had been allowed to multiply for two or three days they underwent a second passage. They were allowed to grow again and subsequently underwent several passages. Generally after a few passages the cells grew together, forming a dense tissue.

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The washings and passages were repeated as often as was necessary, and in this way it was possible to study the morphological changes in cells belonging to a known strain.

The experiments were carried out with ameboid and round connective tissue cells of the chick. The primary cultures were obtained from cultures of connective tissue, which were more than two months old. The old cultures were selected because it seemed that these cells had adapted themselves to their new form of life and that they could stand the washings and the passages better than the cells contained in the younger cultures. All the experiments in which the cells were taken from cultures more than sixty days old were successful. Two experiments only will be described.

PURE CULTURE OF AMEBOID CELLS.

Experiment 1. Primary Culture.—On March 20, 1912, a fragment of plasma at the peripheral part of a culture of heart sixty-three days old was resected. The fragment contained large ameboid cells only. Each cell could be seen individually. No round cells or spindle cells were present. The fragment was washed and put in a new medium. On March 21 no ameboid cells had passed into the new medium, but the number of cells contained in the old fragment of plasma had markedly increased.

First Passage.—On March 21 the culture was washed in Ringer's solution and put in a new medium. On March 22 the cells contained in the old plasma had increased so much that a real tissue was formed, but no ameboid cells were in the new plasma.

Second Passage.—On March 25 the area covered by the cells had become very large. The culture was therefore divided into two fragments (culture 1196-1 and culture 1196-2).

1. Culture 1196-1.—On March 26 the original fragment of plasma was surrounded by a large number of active ameboid cells. On March 28 the area covered by the cells had more than doubled. It was divided into two pieces and washed.

Third Passage.—One of the pieces was put in new plasma on March 28. On March 30 the culture had increased greatly in size and was again divided into two parts and washed.

Fourth Passage.—One of the pieces was put in new plasma on March 30, and grew abundantly.

On April 3, 6, 9, 12, 13, and 15, the culture underwent its fifth, sixth, seventh, eighth, ninth, and tenth passages. During that period, the growth, which was very luxuriant, was composed only of ameboid cells, and these were similar to those of the original culture.

After April 18 there was a very abundant growth of ameboid cells. On April 22 the culture was divided into two parts, and then underwent its twelfth

passage. The cells grew rapidly and on April 25 a thirteenth passage was made. On April 26 the cultures were composed of a mass of dense tissue, from the peripheral part of which radiated many ameboid cells.

2. *Culture 1196-2*.—On March 26 large ameboid cells, disposed in short columns, grew in the new medium. On March 28 the old plasma was surrounded by an immense number of cells covering a large area. The culture was divided into two parts and washed.

Third Passage.—On March 28 one of the parts was put in a new medium, and a very large number of ameboid cells, the pseudopodia of which were very short, grew in the new plasma. On April 1 the culture was divided into two parts.

Fourth Passage.—On April 1 one of the parts was put on a piece of silk veil and in a new medium. On April 2 the ameboid cells had covered a large area of the veil which was then divided into two parts.

Fifth Passage.—On April 2 one of the parts was put in new plasma, and on April 4 thick columns of ameboid cells had grown into the new medium from the periphery of the silk veil.

On April 5, 6, 9, and 12, the culture underwent its sixth, seventh, eighth, and ninth passages. The plasma contained in and around the silk network became denser, and the growth of the cells slower. After the tenth passage on April 15 chains of elongated cells appeared in the new plasma. No ameboid cells were observed.

On April 17, 20, 23, and 26, the culture underwent its eleventh, twelfth, thirteenth, and fourteenth passages. The growth was very slow and was composed only of polygonal and fusiform cells. No ameboid or round cells could be seen.

PURE CULTURE OF ROUND CELLS.

Experiment II. Primary Culture.—On April 1, 1912, a culture of connective tissue that had grown very actively for seventy-two days was selected. The central part was composed of a dense tissue surrounded by elongated cells. In the peripheral part of the plasma many round cells were scattered. With a cataract knife, the central portion was removed, leaving the crown of round cells. Microscopical examination showed that no spindle cells had been left. On April 3 the number of the round cells had increased enormously, but no fusiform cells had appeared.

First Passage.—On April 4 the culture was washed in Ringer's solution and put in a new medium. On April 5 the cells multiplied very rapidly; on April 6 a few spindle cells appeared; and on April 7 a large number of fusiform cells were scattered through the new medium.

On April 8 the culture underwent its second passage, and on April 9 the fusiform cells had increased greatly in number. The culture was divided into two parts on April 10.

After the third passage, on April 10, the new tissue increased in density.

On April 13, 16, 18, 22, and 24, the cultures underwent their fourth, fifth, sixth, seventh, and eighth passages. They were composed of a dense mass of tissue from which radiated a crown of elongated cells. There were no round or ameboid cells.

SUMMARY.

In experiment I a group of ameboid cells was isolated from a culture of cardiac muscle sixty-three days old, and cultivated in plasma. After several passages, they formed a dense tissue from which ameboid cells radiated. The culture was divided into two parts. The part cultivated in plasma alone kept its morphological characters and continued to produce ameboid cells. The part cultivated upon silk in plasma became modified; the cells lost their ameboid characters, and were transformed into large elongated cells which were united in chains, or interlaced to form a network.

In experiment II the round cells taken from a culture of connective tissue seventy-four days old multiplied rapidly. They transformed themselves into elongated cells and produced, after a few passages, a mass of dense connective tissue. From the tissue a large number of elongated cells were constantly growing.

In both experiments the tissues originated from the ameboid or round cells extirpated from cultures that were sixty-three and seventy-four days old respectively. These cultures were still growing actively thirty and forty days later; that is, more than one hundred days after the extirpation of the original fragments from the organism.¹

These experiments show that from old cultures it is possible to isolate and propagate cells that belong to a definite type. A tissue, formed by a pure strain of cells, can be obtained in this way, and this new method may be of value in cytological investigations.

¹ The cultures were still living actively on June 2, 1912.

THE INFLUENCE OF ISOAGGLUTININS ON THE FINAL RESULTS OF HOMOPLASTIC TRANS- PLANTATIONS OF ARTERIES.*

By RAGNVALD INGEBRIGTSEN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATES 16 AND 17.

Several investigators have reported that the functional results after homoplastic transplantation of arteries are excellent. But the microscopical examination of the transplanted segment, when made a long time after the operation, has given conflicting impressions as to the value of the transplantation. In several cases Carrel¹ found that after three to five months, not only the elastic framework, but even the muscular fibers of the median coat were in good condition.² Borst and Enderlen,³ on the other hand, observed a disappearance of the muscular fibers and a substitution of the transplanted segment by the connective tissue of the new host, just as in heteroplastic transplantation. Yamanouchi also found a degeneration of the segment when this was examined seventy-four days after the operation.

It is, of course, possible that differences in the technique of these operations may be responsible for the lack of uniformity in the results. But it is also possible that biological differences in the two animals concerned in each experiment were in some cases so great that they prevented the survival of the transplanted pieces.

It is not yet known why homoplastic transplantation does not usually succeed as well as autoplasic. But in the experiments which

* Received for publication, April 23, 1912.

¹ Carrel, A., *Jour. Exper. Med.*, 1910, xii, 460.

² Capelle, A., *Berl. klin. Wchnschr.*, 1908, xlv, 2012. Capelle, Stich, Ward, and Wood had corresponding results, but their examinations of the segment were made within a somewhat shorter interval after the implantation.

³ Borst and Enderlen, *Deutsch. Ztschr. f. Chir.*, 1909, xcix, 54.

are published here, I have attempted to solve part of the problem, and even if the result is a negative one, it may still be of some interest.

In animals of the same species normal hemolysins or cytolysins have not been found, which may explain the biological differences in the individuals, and be made responsible for the disintegration and death of the homoplastic transplanted piece of tissue. We may conceive of the development of immune bodies under these conditions, but there is no evidence of their existence. Agglutination is the only serological reaction that is normally present between the serum of certain individuals and the red blood cells of others. This phenomenon has been rather thoroughly studied during the last few years, and the importance of the isoagglutinins in practical medicine has been increased by the development of direct transfusion of blood from man to man, as a routine measure.

Normal isoagglutinins of human red blood cells were discovered independently by Landsteiner⁴ and Shattock in 1900, and Landsteiner showed that all specimens of human blood could be divided into three groups according to the way in which they agglutinate. To these three groups a fourth was added by other investigators.⁵ Landsteiner then assumed that the four groups were explained by the existence of two different agglutinins, one of which was present in group 2, another in group 3, both in group 1, and none in group 4.

Epstein and Ottenberg⁶ and von Dungern and Hirschfeld have discovered the interesting fact that the isoagglutinins are transmitted by heredity and follow the Mendelian law. Grouped isoagglutinins have been found also among lower animals. Ottenberg and Friedmann⁷ found grouped agglutinins in rabbits and cattle.

I have tried now to determine if the isoagglutinins exert any influence upon the final results of homoplastic transplantation of arteries.

⁴Landsteiner, K., *Wien. klin. Wchnschr.*, 1901, xiv, 1132; *München. med. Wchnschr.*, 1902, xlix, 1905.

⁵Landsteiner, K., and Leiner, K., *Centralbl. f. Bakteriol., Orig.*, 1905, xxxviii, 548.

⁶Epstein, A. A., and Ottenberg, R., *Proc. N. Y. Path. Soc.*, 1908, viii, 117; *Arch. Int. Med.*, 1909, iii, 467.

⁷Ottenberg, R., and Friedmann, S. S., *Jour. Exper. Med.*, 1911, xiii, 531.

First, I tried to find isoagglutinins in dogs, but without success. The same result is reported by Hektoen.⁸ After several fruitless experiments, that were hampered by the tendency of dog blood to lake, I began to work with cats.

The blood of forty cats was examined in four series, each of which contained ten animals, and the serum of each cat was tested against the red cells of all the other cats in the same group. In these experiments the technique described by Epstein and Ottenberg was used. One volume of a 5 per cent. suspension of erythrocytes in isotonic salt solution was mixed with two volumes of serum in capillary pipettes. The pipettes were incubated at 38° C. for two hours and then put in an ice box for twenty-four hours. Each series was in that way examined twice. The results are recorded in the following tables.

		Serum.									
		1	2	3	4	5	6	7	8	9	10
Blood Cells.	1	—	—	—	—	—	+	—	—	—	—
	2	—	—	—	—	—	+	—	—	—	—
	3	—	—	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	+	—	+	—	—
	5	—	—	—	—	—	—	—	—	—	—
	6	—	—	—	+	—	—	—	+	—	—
	7	—	—	—	—	—	+	—	—	—	—
	8	—	—	—	—	—	+	—	—	—	—
	9	—	—	—	—	—	+	—	—	—	—
	10	—	—	—	—	—	+	—	—	—	—

		Serum.									
		11	12	13	14	15	16	17	18	19	20
Blood Cells.	11	—	—	—	—	—	—	—	—	—	—
	12	—	—	—	—	—	—	—	—	—	—
	13	—	—	—	—	—	—	—	—	—	—
	14	—	—	—	—	—	—	—	—	—	—
	15	—	—	—	—	—	—	—	—	—	—
	16	—	—	—	—	—	—	—	—	—	—
	17	—	—	—	—	—	—	—	—	—	—
	18	—	—	—	—	—	—	—	—	—	—
	19	—	—	—	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	—	—

⁸ Hektoen, L., *Jour. Infect. Dis.*, 1907, iv, 297.

		Serum.									
		21	22	23	24	25	26	27	28	29	30
Blood Cells.	21	—	—	—	—	—	—	—	—	—	—
	22	—	—	—	—	—	—	—	—	—	—
	23	—	—	—	—	—	—	—	—	—	—
	24	—	—	—	—	—	—	—	—	—	—
	25	—	+	—	—	—	—	—	—	—	—
	26	—	—	—	—	—	—	—	—	—	—
	27	—	—	—	—	—	—	—	—	—	—
	28	—	—	—	—	—	—	—	—	—	—
	29	—	+	—	—	—	—	—	—	—	—
	30	—	—	—	—	—	—	—	—	—	—

		Serum.									
		31	32	33	34	35	36	37	38	39	40
Blood Cells.	31	—	—	—	—	—	+	—	—	—	—
	32	—	—	—	—	—	—	—	—	—	—
	34	+	—	—	—	—	+	—	—	—	—
	35	—	—	—	—	—	+	—	—	—	—
	36	—	—	—	—	—	—	—	—	—	—
	37	—	—	—	—	—	—	—	—	—	—
	38	+	—	—	—	—	+	—	—	—	—
	39	—	—	—	—	—	—	—	—	—	—
	40	—	—	—	—	—	—	—	—	—	—

The reaction was recorded as positive (+) only when, after macroscopical inspection, the agglutination was plainly seen on the first as well as on the second examination. In two or three cases the agglutination was very strong and occurred some minutes after the mixture had been made; but usually it was not marked until the next day; *i. e.*, after twenty-four hours in the ice chest.

As may be seen in the tables, the instances of agglutination are distributed among the cats without the slightest possibility of arranging the individuals in groups having the same characteristics.

In some of these cats a segment of the carotid artery (two centimeters long) of one was transplanted to the carotid of the other. In these operations I followed closely the technique described by Carrel. Fourteen transplantations were made; in five of them there was a positive agglutination test between the two animals, and in nine of them the agglutination test was negative. After the segment had been taken out, it was washed in Ringer's solution and then kept in vaselin at 38° C. until it was put in the new host, one half to three fourths of an hour later.

The operation and the convalescence in these fourteen cases were successful and uneventful. Infection and bleeding did not occur.

All of the fourteen transplanted segments were removed from the cat three months after they had been transplanted and were then examined microscopically. Of the five cats in which the agglutination test was positive, three had ideal functional results, and of the nine animals in which the agglutination tests were negative five had ideal functional results with good pulsation and no thrombosis of the artery. In the other six cases there was thrombosis of the artery and of the transplanted segment.

That thrombosis occurred in such a large percentage of the cases is not astonishing and is explained perfectly upon mechanical grounds. As the carotid artery in my cats was only one millimeter in diameter, the slightest stenosis or lack of approximation in suturing produced complete thrombosis of the whole lumen of the vessel.

I shall describe only the experiments in which the functional results were good, as it is not possible to draw conclusions of value from the microscopical examination of thrombosed arteries. Although the muscular fibers with which we are here especially concerned are exposed in thrombosed arteries to the blood serum of the new host and for this reason may show changes due to chemical influences or lack of proper nourishment, I have discarded them in these experiments since the muscular fibers in a thrombosed artery are inactive, and also are under abnormal conditions, both biologically and functionally.

INTERAGGLUTINATION POSITIVE.

Experiment 1.—January 25, 1912. From cat 25 a segment of the carotid artery 2 cm. long was taken and transplanted to the carotid artery of cat 22. The serum of cat 22 agglutinated the red cells of cat 25.

April 22. At the operation good pulsation was present in the carotid but there were some adhesions between the segment and the pneumogastric nerve. The segment was removed.

Microscopical Examination.—Adventitia and periadventitia were thickened, and richly vascularized. The median coat was of normal thickness, the muscular fibers and their nuclei were present in normal number and their appearance was quite normal. With Weigert's stain the elastic framework appeared normal. The internal coat was not thickened (figure 1).

Experiment 2.—December 14, 1911. A segment of the carotid artery of cat 1 was taken and transplanted to the carotid artery of cat 6. The serum of cat 6 agglutinated strongly the red cells of cat 1.

March 16. At the operation good pulsation was present in the carotid but the transplanted segment was bound by slight adhesions to the surrounding tissues. The segment was taken out.

Microscopical Examination.—The adventitia was thickened, richly vascularized, and contained several more lymphocytes than usual. The median coat was only half its normal thickness. All the muscular fibers and their nuclei had disappeared and the elastic framework layers were lying close to each other; the median coat contained nothing but elastic fibers. The internal coat was very much thickened (figure 4).

Experiment 3.—December 14, 1911. A segment of the carotid artery of cat 6 was transplanted to the carotid of cat 8. The serum of cat 6 agglutinated strongly the red cells of cat 8, and the serum of cat 8 agglutinated the cells of cat 6 only a little less strongly.

March 17, 1912. At the operation good pulsation was present in the carotid. There was no thrombosis, but adhesions were found between the segment and the internal jugular vein and the pneumogastric nerve. The segment was taken out.

Microscopical Examination.—The adventitia was thickened, and richly vascularized. The median coat was of normal thickness. In its outer layers the muscular fibers and their nuclei were normal in number and appearance. In its inner portion the fibers seemed a little atrophied and had taken only slightly the protoplasmic stain of Van Gieson. The nuclei had largely disappeared. There was no increase of the connective tissue; the elastic framework was normal; and the internal coat was slightly thickened.

INTERAGGLUTINATION NEGATIVE.

Experiment 4.—December 9, 1911. A piece of the carotid artery of cat 18 was transplanted to cat 19. Between these two cats the agglutination test was negative.

March 8, 1912. At the operation good pulsation was present in the carotid but there were adhesions to the vagus and the internal jugular vein. The segment was taken out.

Microscopical Examination.—The adventitia was considerably thickened, and consisted partly of thick hyaline connective tissue bundles. Around the small vessels, aggregations of lymphocytes were seen. The median coat was thin and flattened. Almost all of the muscular fibers and their nuclei had disappeared. The elastic framework layers were lying close to each other, and the internal coat was covered by a thin layer of amorphous fibrin.

Experiment 5.—January 4, 1912. A piece of the carotid artery of cat 4 was transferred to cat 9. The agglutination test between these two cats was negative.

April 3. At the operation good pulsation was felt in the carotid but there were adhesions to the surrounding tissue. The segment was taken out.

Microscopical Examination.—The adventitia was thickened and richly vascularized. The inner layers were sclerotic. The median coat was a little thinner than normally. In the inner portion the muscular fibers had disappeared. In the outer portion they were still present in almost normal number.

The fibers were atrophied, their nuclei were not very distinctly stained, and their outlines were not sharp. The elastic framework was normal. In the internal coat there were three to four layers of connective tissue cells; in other places the internal coat was not thickened.

Experiment 6.—January 3, 1912. A piece of the carotid artery of cat 11 was transplanted to cat 12. The agglutination tests between these animals were negative.

April 5. At the operation good pulsation was felt in the carotid but there were adhesions between the segment and the surrounding tissue. The segment was taken out.

Microscopical Examination.—The adventitia was much thickened and richly vascularized in the outer layers; the inner layers were partly sclerotic. The median coat was of normal thickness. In the outer layers there were a normal number of muscular fibers, but in the inner layers these had partly disappeared. The elastic framework was normal. The internal coat was a little thickened and had three to four layers of connective tissue cells.

Experiment 7.—January 9, 1912. A piece of the carotid artery of cat 14 was transplanted to the carotid artery of cat 15. The agglutination tests between these two animals were negative.

April 14. At the operation good pulsation was felt in the carotid but there were adhesions to the internal jugular vein and the pneumogastric nerve. The segment was taken out.

Microscopical Examination.—The adventitia was greatly thickened. The median coat was thin and flattened and consisted almost exclusively of the elastic framework, the muscular fibers having disappeared for the greater part. The internal coat was slightly thickened by the development of very thin elastic fibers.

Experiment 8.—January 11, 1912. A piece of the carotid artery of cat 24 was transplanted to the carotid artery of cat 26. The agglutination tests between these two animals were negative.

April 13. At the operation good pulsation was felt in the carotid but there were adhesions to the surrounding tissue. The segment was taken out.

Microscopical Examination.—The adventitia was thickened, but the median coat was thinner than normally. Many of the muscle fibers had disappeared; the elastic framework was normal, and the internal coat was a little thickened.

The sections of the arteries were stained with Van Gieson's stain, with Weigert's elastic tissue stain, and with hematoxylin and eosin.

Results.—In all of the transplanted arteries examined three months after transplantation, there was a thickening of the external coat and a persistence of the elastic fibers of the median coat. Further I found in most of the cases a more or less pronounced thickening of the internal coat.

Very often the external coat had thickened until it was three times its normal size. The new layers of connective tissue had the appearance of cicatrizing tissue; the external coat was somewhat infil-

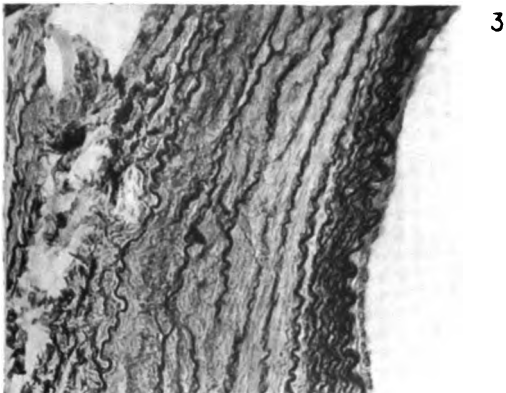
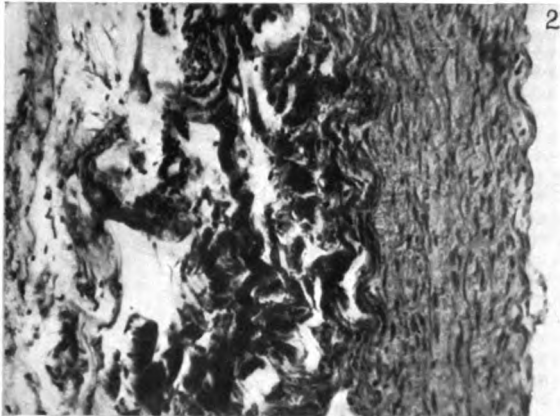
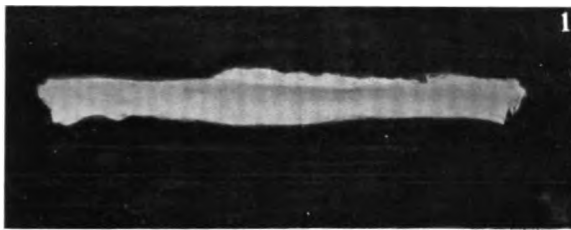
trated by round cells and many small vessels were seen, in the walls of which lymphocytes were aggregated. The bundles of connective tissue were thick, and the inner layers were partly hyaline and sclerotic.

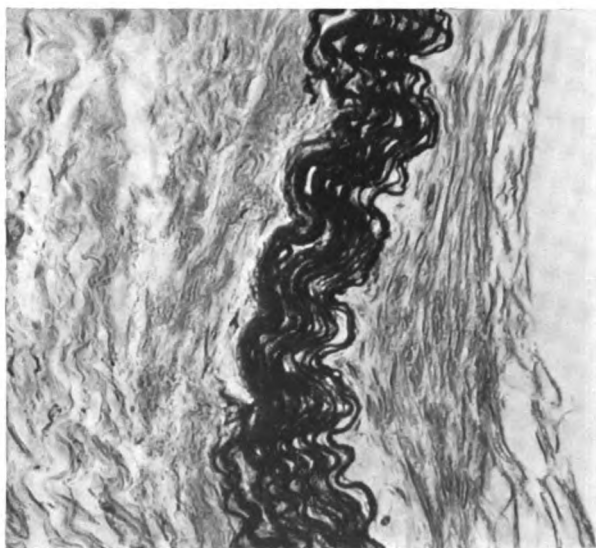
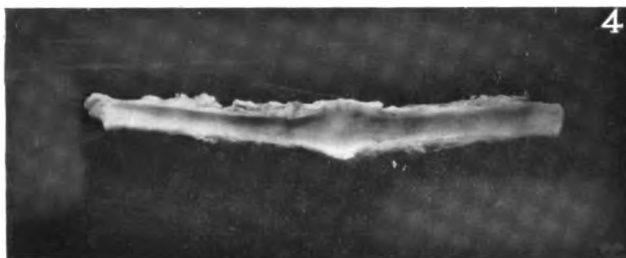
The elastic framework of the median coat was always found in good condition; splitting of the fibers or the formation of granules was not observed. The appearance of sections stained by Weigert's elastic tissue stain, however, was influenced by the presence or absence of the muscle fibers. If all of the muscle fibers had disappeared, the elastic fibers and layers were pressed together and lay closely to each other without any tissue between them. The median coat as a whole was very thin (figure 2). If, on the other hand, the muscle fibers were present, the elastic framework had quite a normal appearance (figure 3). In no case was a proliferation of cells of any kind, or a formation of new tissues within the median coat observed. In most of the cases the smooth muscle fibres of the median coat had partly disappeared.

There were some few features of interest. In the first group of transplantations (three experiments with positive interagglutination) the muscle fibers were apparently quite normal in appearance and number in experiment 1, and also perhaps to a less extent in experiment 3, while in experiment 2 these fibers had completely disappeared (figures 1, 2, and 3). Experiments 1 and 2 are within the same group and yet are at opposite extremes as far as the muscular fibers are concerned. Experiment 2 was the one in which the isoagglutination was strongest, but that this coincides with the most complete disappearance of muscular fibers must be considered accidental, because the other two experiments in the same group show muscular fibers in a very good condition.

In the second group (five experiments, in which interagglutination was negative) the results in the different experiments corresponded more closely. Here most of the muscular fibers, but not all had disappeared, and as a general feature it was observed that they had disappeared in the inner layers of media, while they were still present in the outer.

The thickening of the internal coat went parallel with the disappearance of the muscle fibers of the median coat; the strongest





increase was seen in experiment 2 where all of the muscle fibers had disappeared. In experiment 1 where all of the muscle fibers were present, the internal coat was thickened but little (figures 1 to 6).

CONCLUSIONS.

The results in the group in which there was interagglutination differ in no striking way from those obtained in the group in which there was no interagglutination. It may be concluded, therefore, that the presence of isoagglutinins is of no importance for the final results of the homoplastic transplantation of arteries. From the conflicting evidence of the survival of muscular fibers in one case and their disappearance in many other cases, it may be concluded further that between animals of the same species there are unknown biological differences that prevent, in most instances, the survival of homoplastic transplanted arteries, although a survival does occur in some cases.

EXPLANATION OF PLATES.

PLATE 16.

FIG. 1. Gross appearance after three months of a segment of a carotid artery transplanted from cat 25 to cat 22. Agglutination test positive.

FIG. 2. Transverse section of the segment. Internal coat not thickened. The median coat has the normal number of muscle fibers and nuclei. The external coat is somewhat thickened. Van Gieson's stain.

FIG. 3. Transverse section of the same specimen. Internal coat slightly thickened here. Weigert's elastic tissue stain.

PLATE 17.

FIG. 4. Gross appearance after three months of a segment of a carotid artery transplanted from cat 6 to cat 1. Agglutination test positive. The suture lines can hardly be seen.

FIG. 5. Transverse section of the transplanted segment. The internal coat is very thick. The median coat is thin without muscle fibers. The external coat is very thick and infiltrated by round cells. Van Gieson's stain.

FIG. 6. Transverse section from the same specimen. Weigert's elastic tissue stain.

STUDIES UPON THE CHARACTERISTICS OF DIFFER-
ENT CULTURE MEDIA AND THEIR INFLUENCE
UPON THE GROWTH OF TISSUE OUTSIDE
OF THE ORGANISM.*

By RAGNVALD INGEBRIGTSEN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

PLATES 45-54.

In a previous article¹ I have called attention to the fact that heat exerts a marked influence upon serum when used as a culture medium. This was observed in cultures in serum agar of the bone marrow of adult guinea pigs. It was supposed that the influence which the heating of the serum exerted upon its value as a culture medium and upon the growth of tissue in it was indicated truly by the rapidity of the emigration of ameboid cells from the piece of tissue. I did not know then whether these cells really multiplied, or whether they only migrated. In order to reach conclusive results it was necessary to carry these experiments further.

The experiments, the results of which I shall give in this paper, were planned to investigate thoroughly the influence of heat upon serum as a culture medium, taking into consideration at the same time the value of different media and the conditions necessary for the growth *in vitro* of adult and embryonic tissue.

To distinguish between emigration and multiplication of cells in a culture may be very difficult. *In vitro* the lymphoid cells show ameboid movements, but motility is not at all limited to these cells, for it may be manifested by every connective tissue cell, even the highly differentiated cells of the cornea. The movements of the connective tissue cells, however, are very slow compared with the rapid movements of the ameboid lymphoid cells, which under the

* Received for publication, June 3, 1912.

¹ Ingebrigtsen, R., *Jour. Exper. Med.*, 1912, xv, 397.

microscope may be seen to change from second to second. Since the first stage of growth is very often only an emigration, it is not permissible to conclude that all the cells that are seen surrounding the original fragment represent a growth of the latter.

Direct observation under the microscope of cells that are dividing is proof, of course, that they multiply, but to make these observations great patience is required, and even with patience one may fail to see cells that are dividing. Another evidence of growth is the occurrence of mitotic figures in emigrated cells that divide indirectly. But for cells that divide amitotically, such as leucocytes, this criterion cannot be used. Mitotic figures found in the original piece itself by making sections of it, as Oppel² did, do not prove that there is growth outside of the organism, for these figures might, of course, be present before the fragment was removed and transplanted to the plasma.

Real growth *in vitro* cannot be acknowledged as proved unless a new tissue that grows from the periphery forms around the original fragment.

This is most easily observed in cultures of certain epithelial organs (hypophysis, thyroid), which form *in vitro* strands, columns, and arches of cells that lie close together, the basal cells of which are inactive and show but little alteration, while the peripheral cells change and multiply continually. The same observation may be made in growing connective tissue (endothelial cells) (figure 13), but here it is not so marked nor so easily confirmed, because the connective tissue cells have motility and are not so liable to keep close together or to form sheets of tissues.

Such a newly formed and growing tissue proves beyond doubt that multiplication of cells has occurred, and the mitotic figures present in this tissue afford additional evidence of growth.

In these experiments I have cultivated adult and embryonic tissues in different media, that is, in autogenic and homogenic plasma, homogenic serum (heated and unheated) plus 2 per cent. agar, homogenic serum, Ringer's solution plus 2 per cent. agar, and in Ringer's solution alone.

²Oppel, A., *Anat. Anz.*, 1912, xl, 464.

The plasma cultures were prepared in the ordinary way described by Carrel and Burrows.³

The cultures in serum and agar and Ringer's solution plus agar were prepared according to the technique described by me in another article.⁴

Cultivation of tissue in serum and in Ringer's solution was carried out in drops hanging from the under surface of cover-slips that were sealed to hollow slides, care being taken that the amount of fluid surrounding each piece of tissue covered it in a very thin film.

Adult Tissue.—From several adult cats, rabbits, and guinea pigs, I cultivated spleen, bone marrow, hypophysis, and thyroid gland. Real growth, that is formation of a new tissue around the original fragment was obtained in practically all experiments when the tissues were cultivated in plasma. Morphologically the organs behaved almost exactly alike whether taken from cats, rabbits, or guinea pigs.

From thyroid and hypophysis there appeared on the second and third days solid columns of epithelial cells that represented in all probability the specific glandular cells of these organs (figures 16 and 24); in some cases long spindle connective tissue cells (figure 15) grew out and the presence of these cells apparently prevented the development of the epithelial structures.

In the first stages of growth of spleen and bone marrow there are always emigrations of ameboid cells, which start moving one or two hours after the beginning of the incubation of the culture. On the third day the ordinary connective tissue cells begin to grow and, after incubation for five or six days, form a new connective tissue that surrounds the original fragment.

While in plasma all the organs of the adult animals examined give rise to real growth, usually of their specific cells, but sometimes only of the connective tissue cells, in not one case have I found real growth of the organs of adult animals when cultivated in the other media mentioned; that is, in Ringer's solution, in Ringer's solution plus 2 per cent. agar, in serum, and in serum plus 2 per cent. agar.

³ Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 387.

⁴ Ingebrigsten, R., *loc. cit.*

In these media the emigration of cells is very often seen, and from lymphatic tissue (bone marrow) in serum plus agar this emigration is very rapid. But the formation of new tissues has not been observed.

Embryonic Tissue.—It is quite different, however, with embryonic tissue which grows well in plasma, but just as well and even better sometimes in some of the other media.

Tissues from chick and cat embryo were examined. The cat fetus was almost at full term when taken from the uterus of the mother. The chick embryos used were fifteen to sixteen days old.

Peritoneum, skin, liver, and thyroid of the fetal cat grew very well in serum agar. They started to grow on the first day and formed around the original fragment during the following two or three days an extensive area of new tissue (epithelial cells from the thyroid, liver, and skin, and connective tissue cells from the peritoneum) (figures 13, 14, 17, 18, 21, and 22). In serum alone these tissues grow very little, a few connective tissue cells only spreading along the cover-glass.

In Ringer's solution and in Ringer's solution plus agar they did not grow at all.

Heart, spleen, skin, and liver were cultivated from the chick embryos fifteen to sixteen days old. These organs grew very well in serum plus agar.

The epithelial cells of the liver and skin grew much more extensively and vigorously in chick serum plus agar than in chick plasma (figures 17 and 18). In serum agar they grew usually in one layer between the serum agar clot and the cover-glass. From the heart and spleen of chick embryos connective tissue cells developed actively in serum agar (figures 1 to 12), but not as well as in plasma. After three to five days the spleen cultivated in plasma showed a wider growth of elongated connective tissue cells, and the area of emigrated cells was larger and denser in the plasma than in the serum agar (figures 11 and 12). Chick heart in plasma gave rise to a dense framework of connective tissue that surrounded the fragment evenly. In serum plus agar the same kind of cells developed but on a smaller scale.

In serum, connective tissue from chick heart grew rather exten-

sively along the cover-glass in a thin layer, but it did not grow so well as in serum agar. In serum, chick liver and spleen showed an emigration of cells, but no growth.

In Ringer's solution and in Ringer's solution plus agar some slight emigration of cells occurred from chick spleen, but these cells lost their ameboid movements within the first twenty-four hours, while the same kind of cells, emigrated from spleen in serum, showed ameboid movements for at least three days (figures 7 and 8). In Ringer's solution and in Ringer's solution plus agar, no growth of the cells from the sixteen day chick embryo has been observed.

I wish to emphasize in this place the differences found between adult and embryonic tissues. The former does not grow at all in any medium but plasma, while embryonic tissue develops vigorously also in serum plus agar.

Furthermore, in Ringer's solution and in Ringer's solution plus agar, I found no growth of the cells from the fifteen to sixteen day old chick embryos.

Even within the embryonic stage there are apparently great differences in the energy of proliferation of cells of different ages. The energy of growth *in vitro* decreases as the age of the embryo increases, and the culture experiments that succeed when performed with a chick embryo that is eight days old may be completely unsuccessful with a chick embryo sixteen days old.

Thus, in an extensive series of experiments, Lewis and Lewis⁵ have cultivated several tissues of chick embryo in various salt solutions. They generally used chick embryos from seven to nine days old, and in some cases embryos that were thirteen to fifteen days old. In their experiments they observed emigration of cells as well as formation of syncytial membranes. Many of their illustrations are rather convincing as to the presence of multiplication among the cells. In a few experiments in which I cultivated in Ringer's solution connective tissue from chick embryos that were six to eight days old, I had corresponding pictures.

Lewis and Lewis, however, express it as their conviction that the

⁵ Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277.

salt solutions utilized by them were protective and not nutrient in action, and this view is certainly correct.

Of the cultures that they prepared, the percentage that was positive decreased as the age of the embryos utilized increased. This varying percentage shows that the very young embryonic tissue has such a tremendous energy of proliferation and is so little dependent on the constituents of the media in which the cells live that they must be considered as constituting a peculiar sort of tissue. The limits within which these salt solutions of Lewis may be varied quantitatively and qualitatively are so extensive, that they cannot be supposed to be of great importance in the metabolism of these cells. By their tremendous energy of proliferation they grow perhaps in spite of the media instead of because of them, and, as Lewis and Lewis themselves say, they grow within the limits determined by the amount of food stored up in the body of each individual cell.

They have, therefore, a comparatively short existence in salt solution, while in nutrient media they live much longer and, as shown by Carrel,⁶ may even be kept in permanent life. There is, therefore, a fundamental difference between the life of very young embryonic tissue in salt solution (a preservative medium) and in plasma (a nutrient medium). If we wish to realize the relationship between these two media, we may compare the growth in salt solution with the pale and whitish buds that grow out of potatoes in the spring, if they are placed in a dark and damp cellar, while the growth in plasma and serum agar resembles the powerful, young green leaves that sprout out above the surface of the soil when the potato is permitted to draw energy and nutrition from light, air, and ground. The life and growth of these young embryonic cells in salt solution can hardly be called a culture in the true sense of this word.

Influence of Heat. Homogenic Serum.—In order to ascertain whether the influence of heat exerted upon serum is of importance, not only for the emigration and survival of ameboid cells but also for the multiplication and the growth of tissue cells, it was necessary to cultivate embryonic tissue in heated and unheated serum, because adult tissues do not grow at all in sera.

⁶ Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516.

Spleen, liver, and skin of chick and cat embryos have been cultivated in heated and unheated homogenic serum plus agar. Spleen of the fetal cat gave only a small growth in serum agar, and was, therefore, not suitable for my purpose. Spleen of a sixteen day chick embryo, on the other hand, grew very well in serum agar and was well adapted for comparative experiments. I found that the growth in unheated serum agar was quantitatively different from that in heated serum plus agar. In the former there appeared after two and three days several elongated cells that formed around the fragment a "corona radiata" that was very similar to that in plasma, while these cells did not develop in heated serum plus agar (figures 10 and 11). In the latter the area of emigrated cells was smaller than in unheated serum plus agar. In these experiments the chick serum had been heated to 52° to 53° C. only. Chick serum cannot be heated beyond that temperature without the occurrence of a precipitation with a subsequent deficit of proteins, and probably salts, in the fluid. The maintenance of the percentage of proteins was, of course, necessary.

The cultivation of epithelial tissues from cat and chick embryo in heated and unheated homogenic serum agar has not given the results that might be expected from the results obtained with chick spleen. In many of the experiments, liver and skin grew better in unheated than in heated serum agar, but in some cases they have grown as well, and in a few cases even slightly better in heated than in unheated serum (cat serum was heated to 58° to 59° C. for half an hour and chick serum heated to 52° C. for half an hour).

The epithelial cells of liver and skin grew very quickly and extensively in one or two layers between the nutrient clot and the cover-glass. The epithelial cells were possibly more dependent upon mere mechanical and physical conditions, as, for example, the space available between the clot and the glass, and the degree of adhesion between these two structures, than were the connective tissue cells, which grew in many different layers through all the surrounding medium and therefore responded more quickly than the epithelial cells to changes in the medium, whatever these might be.

This possibly explains also why embryonic epithelial cells grow better in serum agar than in plasma; for the epithelial cells have a

tendency to spread rapidly along a smooth surface and find better conditions between the serum agar clot and the cover-glass than between the plasma clot and the glass, since the plasma adheres very closely to the glass.

Heterogenic Serum.—In agar plus serum from cats, dogs, and rabbits, I have cultivated in these experiments the heart of a sixteen day old chick embryo.

In the plasma from cats, dogs, and rabbits, a piece of chick heart gave rise to the growth of a connective tissue framework that surrounded the fragment evenly but not densely. In serum agar from these same animals a piece of chick heart grew somewhat differently, a thin layer of spindle connective tissue cells spreading from the fragment as a sheet of tissue or growing out in small bushes or bundles.

A comparison of the quantitative growth can easily be made. I found when comparing the growth of chick heart in unheated and heated heterogenic sera plus agar that heated heterogenic serum is a better culture medium than unheated. On the third day all the cultures examined showed more extensive growth in heated serum plus agar than in unheated serum, and the cells of the former were in a better condition.

Sera of dogs, cats, and rabbits are all hemolytic for the red blood corpuscles of chickens, and it is to be expected that the improvement as a culture medium, obtained by heating the serum, is due to the destruction of its hemolytic powers. Whether this is the whole explanation or whether there are other factors to be considered (specific cytolysins), further experiments will perhaps reveal.

In several series of experiments I have determined the relative hemolytic power of heterogenic sera against chicken and guinea pig blood cells, and then, by preparing cultures from the tissues of the animal that supplied the blood cells in the hemolysis experiments (chicken and guinea pig), I have tried to determine whether there is an inverse ratio between the hemolytic power of a heterogenic serum and the rate of growth in it. Plasma was also used.

Chick heart cultivated in agar plus serum from cats, dogs, and rabbits, after the relative hemolytic powers of these sera for

chick red cells had been determined to be 7.7:7.1:2.0, showed that the areas of growth, measured by the micrometer on the third day in the three sera had the relationship of 3:5:12. The same inverse ratio between the hemolytic powers of heterogenic serum and the growth in it was found when guinea pig spleen and bone marrow were cultivated in agar plus serum of rabbits, dogs, and cats. The limits of the growth in these cases were not determinable with exactness, and I am, therefore, unable to give figures.

In heterogenic plasma, on the other hand, this inverse ratio between the hemolytic power and the extent of the growth in it was not constant. The growth of chick heart in cat plasma, for instance, is better than in dog plasma, even if the relative hemolytic power of cat serum and dog serum for chick blood cells is indicated by 7.7:7.1.

This seems to indicate that the character of the fibrin framework in the plasma clot is of some importance for the growth of cells in it, and that perhaps the structure of the fibrin framework is able to modify the growth even as much as chemical and biological differences are able to.

I have, therefore, started a study of the fibrin framework in different plasmas. The results of these studies are not yet conclusive, but I wish to state that small differences between the characters of the framework of plasma from different species are present. A detailed report of these investigations will be given in another article.

CONCLUSIONS.

1. There is a great difference between embryonic and adult tissue as far as their growth outside of the organism is concerned. Adult tissue grows only in plasma. Embryonic tissue grows also very well in serum and serum plus agar. In Ringer's solution and in Ringer's solution plus agar no growth occurs, whether embryonic or adult tissue is employed; survival and emigration of cells are seen to some extent.

2. For the growth of connective tissue cells of chick embryo, unheated homogenic serum is a better culture medium than heated serum. The growth of epithelial cells is not thus influenced.

3. Heated heterogenic serum is a better culture medium for growth of embryonic connective tissue cells than unheated.

4. There is an inverse ratio between the hemolytic power of heterogenic sera and the extent of growth of tissue in them. This inverse ratio is not found in heterogenic plasmas.

EXPLANATION OF PLATES.

PLATE 45.

Camera lucida drawings made on the third day of incubation of cultures in different media of the spleen of a sixteen day chick embryo. The drawings show the relative extent of the emigration of cells and the growth of connective tissue cells. Growth of these cells occurred only in figures 5 and 6 and is indicated in these figures by the *dark* grey area surrounding the black central spot (the original piece of tissue). The *light* grey area represents the emigrated cells.

- FIG. 1. Chick spleen in Ringer's solution.
- FIG. 2. Chick spleen in Ringer's solution plus 2 per cent. agar.
- FIG. 3. Chick spleen in serum.
- FIG. 4. Chick spleen in heated serum plus 2 per cent. agar.
- FIG. 5. Chick spleen in unheated serum plus 2 per cent. agar.
- FIG. 6. Chick spleen in plasma.

PLATE 46.

Photograph of cells that had emigrated and grown out of pieces of spleen of a sixteen day old chick embryo. The pieces had been incubated in different media for three days. The relative extent of the growth is shown in figures 1 to 6.

- FIG. 7. Chick spleen in Ringer's solution.
- FIG. 8. Chick spleen in Ringer's solution plus agar.
- FIG. 9. Chick spleen in serum.
- FIG. 10. Chick spleen in heated serum plus 2 per cent. agar.
- FIG. 11. Chick spleen in unheated serum plus 2 per cent. agar.
- FIG. 12. Chick spleen in plasma.

Notice the presence of elongated cells in figures 11 and 12 only. Further, note that ameoboid movements are still present on the third day in serum (figure 9), while they stopped within twenty-four hours in Ringer's solution and in Ringer's solution plus agar (figures 7 and 8). Figures 7 to 9 are from photographs of the fresh unstained cells. Figures 10 to 12 are from cells stained with hematoxylin.

PLATE 47.

FIG. 13. A three day culture of peritoneum of fetal cat, in agar plus unheated serum.

FIG. 14. A high power magnification of one of the cells in mitotic division.

PLATE 48.

Five day cultures in plasma of thyroid gland, from an adult cat.

FIG. 15. The development of elongated connective tissue cells.

FIG. 16. Epithelial cells (another culture). No connective tissue cells.

PLATE 49.

A three day culture of liver from a sixteen day old chick embryo.

FIG. 17. In serum agar.

FIG. 18. In plasma.

PLATE 50.

A three day culture of corium, in serum agar, from a sixteen day old chick embryo.

FIG. 19. High power magnification.

FIG. 20. Low power magnification.

PLATE 51.

FIG. 21. A five day culture in plasma of adult guinea pig bone marrow. Elongated connective tissue cells.

FIG. 22. A three day culture of liver, in serum plus agar, from a fetal cat.

PLATE 52.

FIG. 23. Connective tissue cells spreading along the cover-glass in a culture in serum of a sixteen day chick heart.

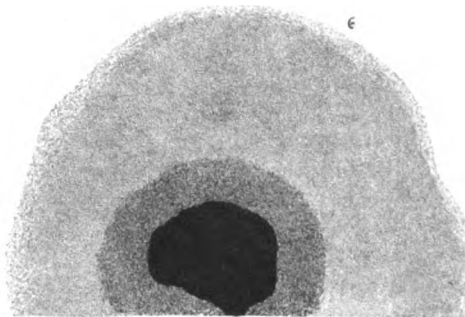
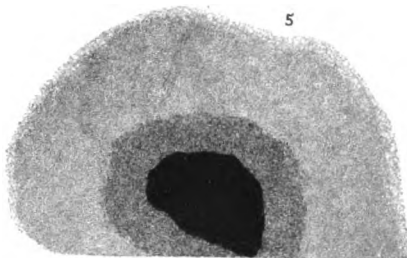
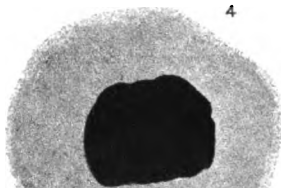
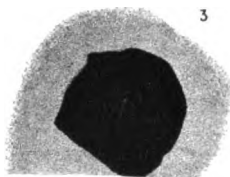
FIG. 24. A five day culture in plasma of hypophysis of an adult cat.

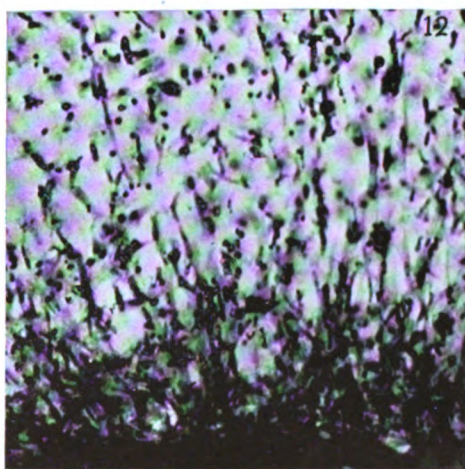
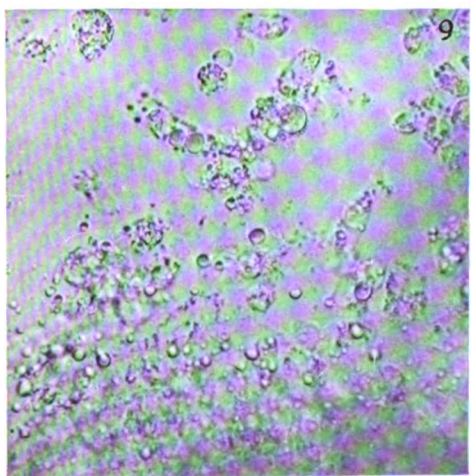
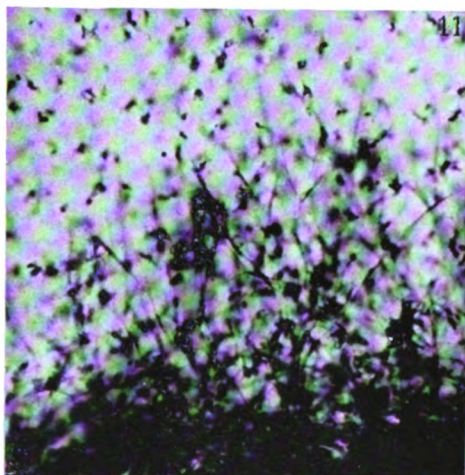
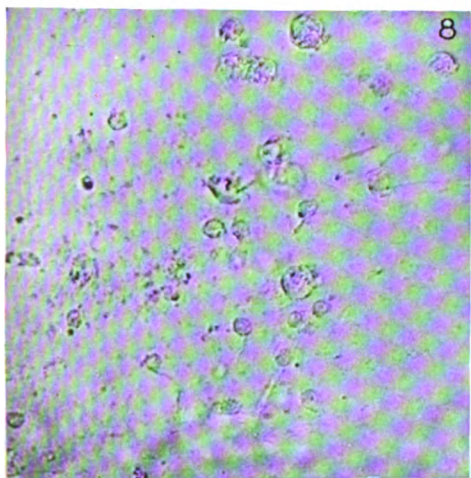
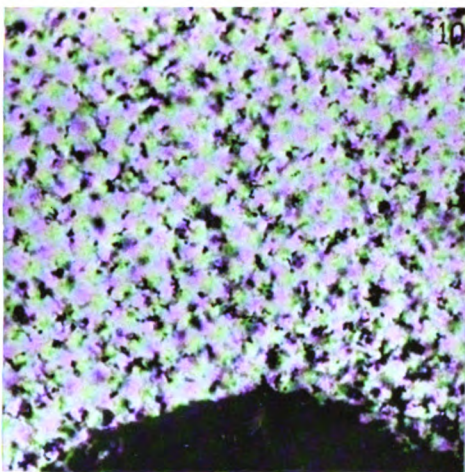
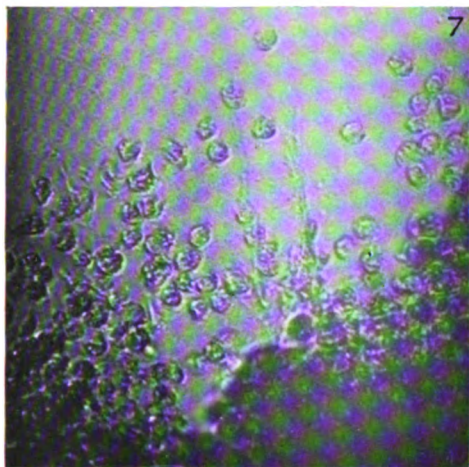
PLATE 53.

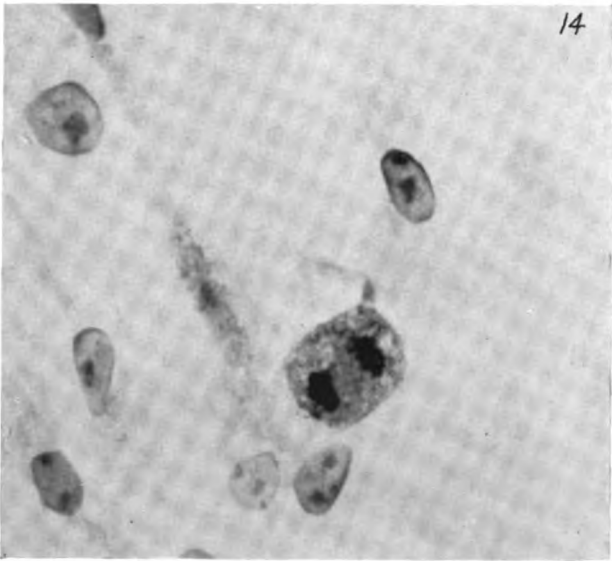
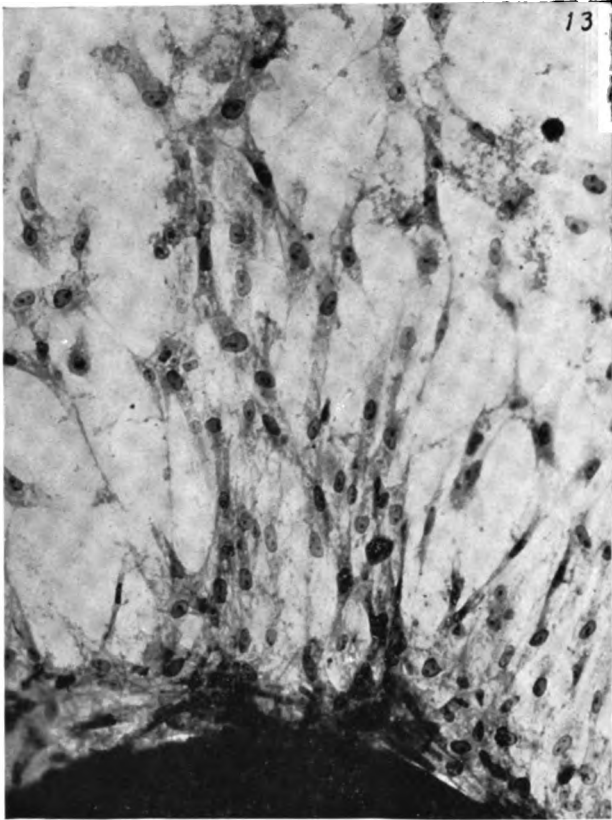
FIG. 25. A three day culture of chick heart (fifteen day chick embryo) in agar plus cat serum. The serum was heated previously for one half hour at 58° C.

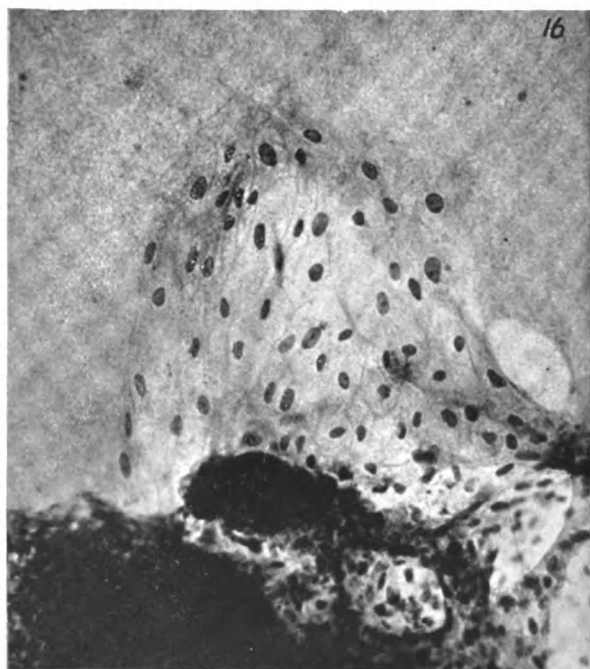
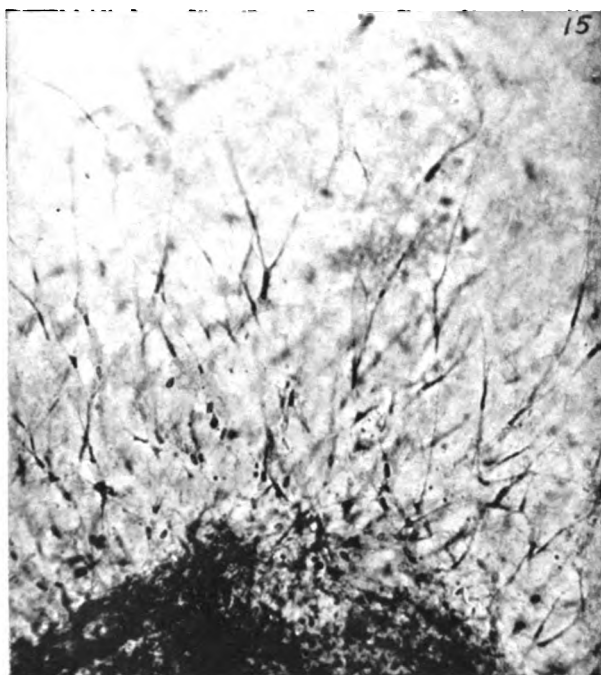
PLATE 54.

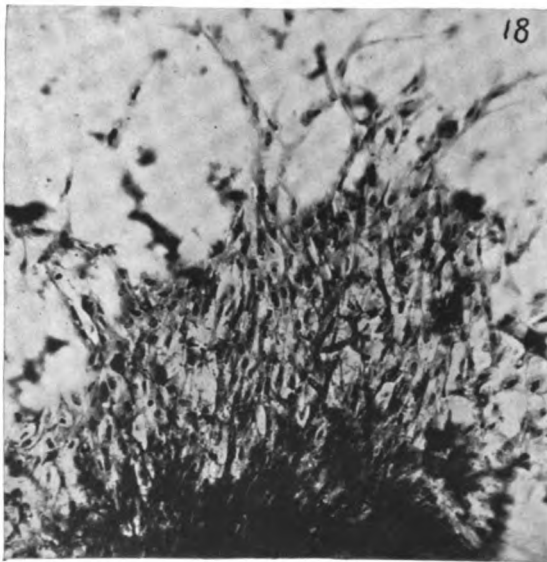
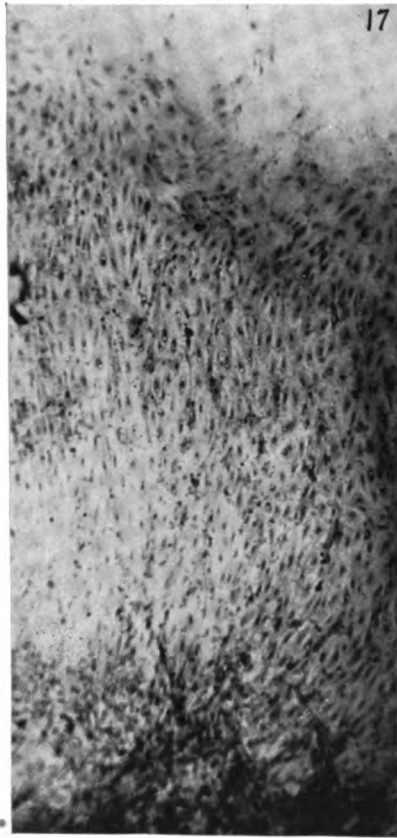
FIG. 26. A three day old culture of chick heart (fifteen day chick embryo) in agar plus cat serum. The serum was unheated.

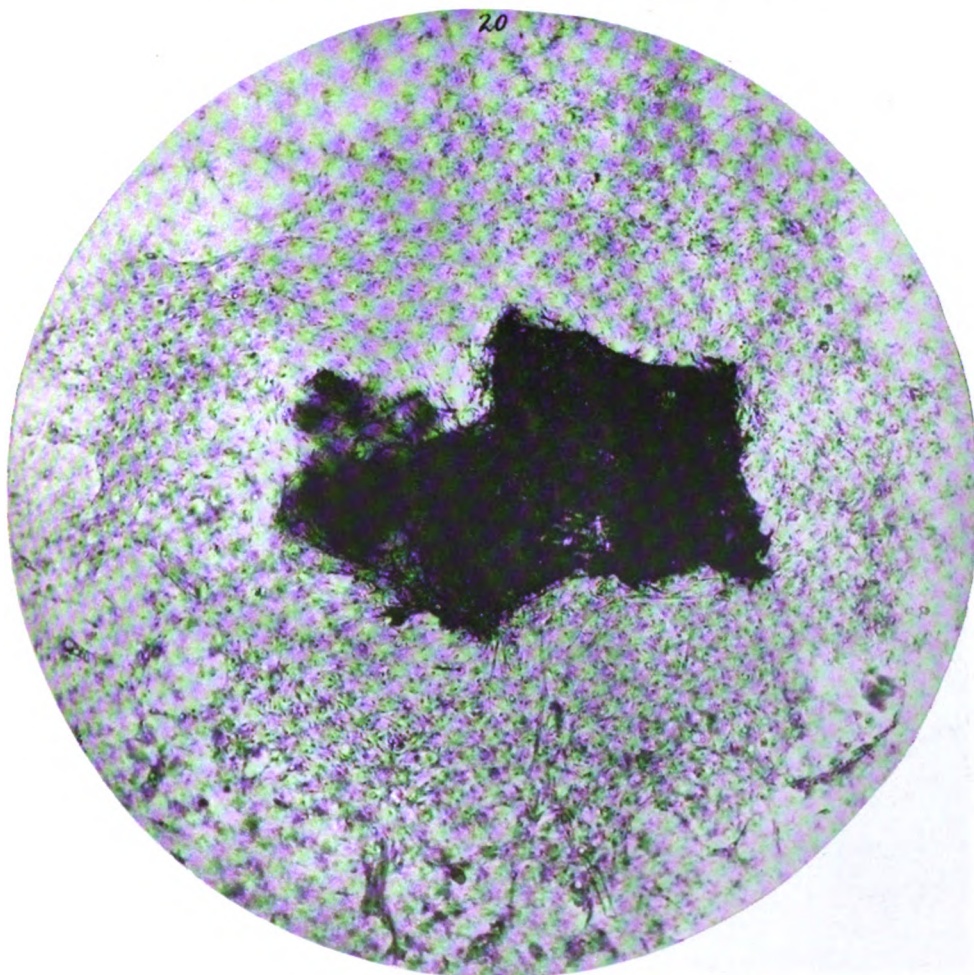
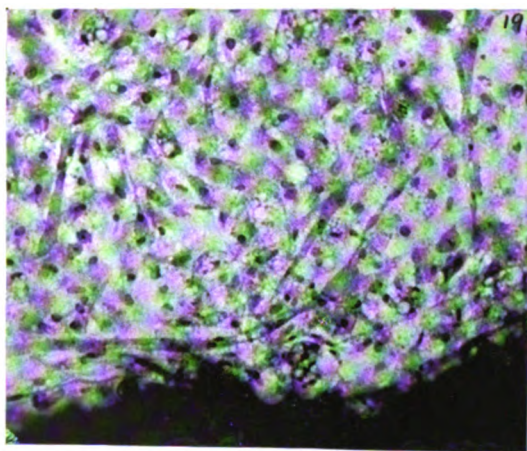


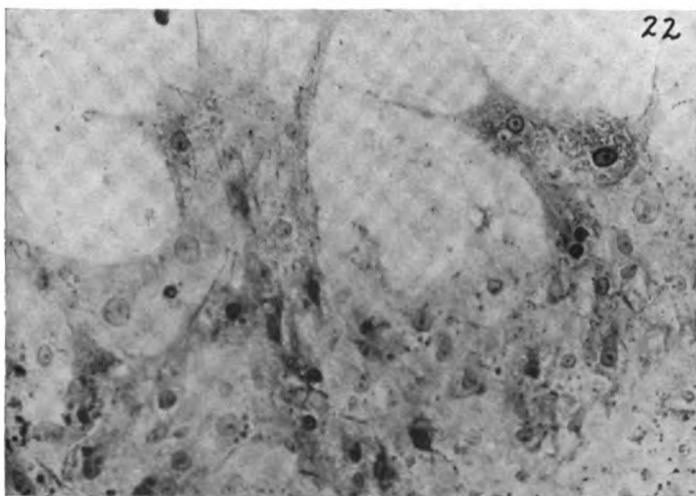
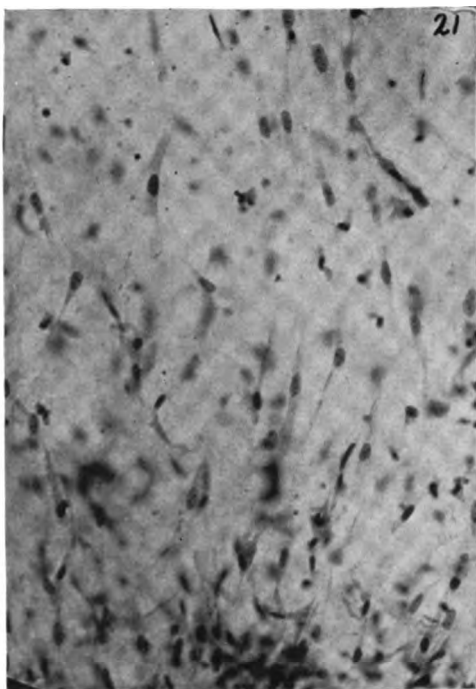


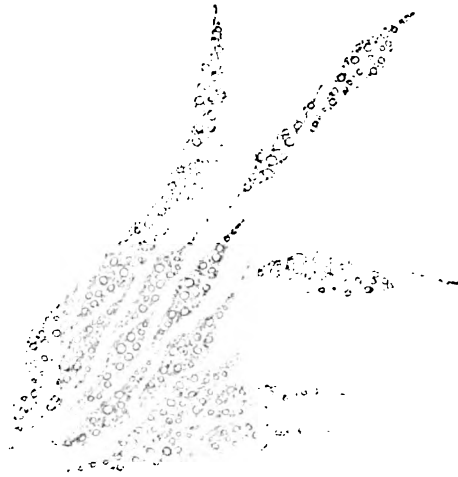












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POTENTIAL DIFFERENCES AT THE JUNCTION OF IMMISCIBLE PHASES.

By REINHARD BEUTNER.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

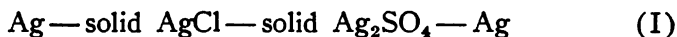
Some four years ago Professor Haber and I¹ investigated the potential differences at the junction of two immiscible phases which are both electrolytic conductors. The usefulness of the results obtained and of the conceptions evolved in this work were confirmed in their application to bio-electric phenomena in a recent investigation carried out by Dr. Jacques Loeb and myself in The Rockefeller Institute for Medical Research.

I. MEASUREMENT AND CALCULATION OF THE E. M. F.'S OF CELLS FORMED OF SOLID SALTS.

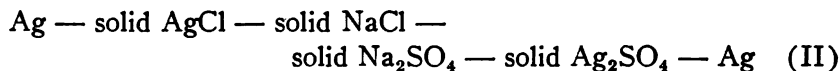
I will first discuss the measurement and calculation of the e. m. f. of cells formed of solid salts while no water is present. The effect of "phase potentials"—that is, potential differences between two electrolytic phases—is seen in this case in a most striking manner. The e. m. f. of cells such as:



or



is strictly zero.² But if sodium salts are introduced into the latter cell, a considerable e. m. f. is produced. For instance, the cell:



¹ F. Haber and R. Beutner, *Annalen der Physik*, **26**, 947 (1908).

² This had been found experimentally by R. Lorenz, before we started our investigation.

gives an e. m. f. of about 0.53 volts. This e. m. f. is much higher than any that could be obtained from a combination of aqueous solutions without metals. The seat of this e. m. f. must be at least partially at the junctions of the salts, since the e. m. f. of (I) is zero, and only the introduction of the sodium salts gives rise to the e. m. f. of 0.53 volt.

I have measured the e. m. f. of (II) by different methods and have always found the same value. At about 300° C. the e. m. f. of such a cell can be easily measured, since solid salts, heated to this temperature, have a fairly good electrolytic conductivity. The resistance is rather high, but with the aid of a good electrometer the e. m. f. can be measured.

How are these potential differences at the junction of salts to be explained? Such conceptions as solution tension and ionic concentration, which are generally applied in the calculation of e. m. f.'s of combinations of aqueous solutions, cannot be used in this case, since the ionic concentration is not known for any solid salt. We should undoubtedly make use of the thermodynamic theory of the galvanic current, which is really the foundation of all physico-chemical theories in this field.

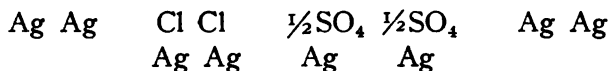
This simple principle, deduced by H. von Helmholtz in 1847, is as follows: The chemical or physical changes, which a current sent through a system brings about in this system, are the cause of an e. m. f., since these changes require the consumption of a certain amount of energy. Work has to be performed, and this work is represented by a special counter e. m. f. which the current has to overcome. There exists an e. m. f. even if there is no current sent through the system from an outside source. If the poles or terminals of the system are short circuited, this e. m. f. will produce a current which causes such changes as will bring the whole system nearer to equilibrium.

The reason why the e. m. f. of system (I) is zero is that no changes of any kind are possible in it. The solid salts are immiscible and cannot react with each other. *All components of the system are in equilibrium with each other however they may be arranged.*

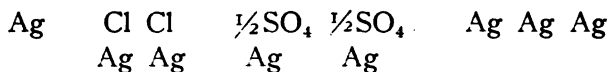
Let us now send an electric current through system (I) from

the left to the right. Metallic silver from the left electrode will be changed into silver chloride, while from the silver sulphate metallic silver will be deposited on the right electrode. Hence at the two electrodes some fresh AgCl is formed and some Ag₂SO₄ disappears. But the total change in the whole system is zero, on account of the electrochemical reaction taking place at the junction of AgCl and Ag₂SO₄, when Ag passes over from AgCl to Ag₂SO₄, so that some fresh Ag₂SO₄ is formed and some AgCl disappears. The junction of AgCl and Ag₂SO₄ is the seat of a potential difference which is equal and opposite to the two potential differences at the poles.

To render the matter clearer, I will write down the changes of the components of the system. Before we pass a current, the system may be represented as follows:



If we now pass a current from the left to the right, the new system produced is as follows:



The total change is therefore:

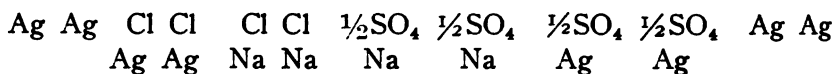
Metallic Ag \rightarrow Ag in AgCl \rightarrow Ag in Ag₂SO₄ \rightarrow metallic Ag.

While there are three potential differences in this cell, their total sum is zero, since all the reactions compensate each other.

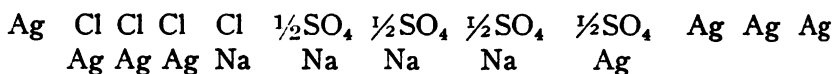
The reason why cell (II) gives an e. m. f. of about half a volt is that its components are not in a state of equilibrium in the same sense as those of cell (I). The solid salts of cell (II) may also be assumed to be immiscible, but equilibrium exists only on account of the order in which the salts are arranged. This arrangement is such that no two salts which are capable of reacting with each other are in direct contact. If, for instance, the solid Na₂SO₄ were removed from its place in system (II) and inserted at another place there would be a reaction between NaCl and Ag₂SO₄, forming AgCl and Na₂SO₄.

Now, what happens if a current is sent through this cell from the left to the right? At the electrodes some new AgCl is formed and some Ag_2SO_4 disappears, just as in cell (I). But in cell (II) the reverse change from AgCl to Ag_2SO_4 (which in cell (I) occurs at the junction of the two salts) does not occur at all, since the two silver salts are separated from each other by the two impermeable sodium salts. Hence the total sum of all the changes produced by the current in the system will not be zero in this case.

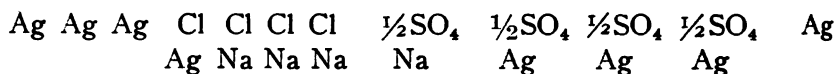
The original system may be represented as follows:



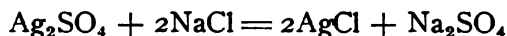
If we pass a current through this system from left to right, the new system produced is as follows:



Or if we pass a current through the original system from right to left, the new system produced is as follows:



Hence in the first case the changes produced by the electric current are the formation of fresh AgCl and fresh Na_2SO_4 and the disappearance of NaCl and of Ag_2SO_4 , while in the second case exactly the opposite chemical change is produced by the passage of the electric current in the opposite direction, so that the cell is reversible. The equation of the reaction is, therefore:



and the direction of the electric current determines whether the chemical reaction occurs from left to right or from right to left in the above equation.

If there is no external source of electric current, and if the silver electrodes of the system are directly connected together by a wire so that the cell is shortcircuited, its e. m. f. must be such that the current produced by it causes formation of those two salts

which are in equilibrium or will not react with each other. These are AgCl and Na_2SO_4 . This means that the silver electrode in contact with Ag_2SO_4 must be the positive pole and this is really the case.

The e. m. f. can be calculated from the free energy of the reaction. For most reactions between solid substances the free energy is practically equal to the heat of reaction. According to Thomsen the heat of this reaction is 24,690 calories or 103,350 watt-seconds or volt-coulombs, and since the reaction is brought about by the passage of $2 \times 96,540$ coulombs, the e. m. f. is $103,350 \div (2 \times 96,540) = 0.535$ volt.

According to Berthelot the heat of reaction is 23,200 calories, from which the e. m. f. is found to be 0.515 volt.

The e. m. f. was measured at two different temperatures, and the following results were obtained:

at 160° 0.532 volt.
at 310° 0.532 volt.

II. RELATION OF THE PHASE JUNCTION TO POTENTIAL DIFFERENCES.

Cells formed of saturated solutions or molten salts behave in a very different way. For instance, let us make a cell in a manner analogous to cell (I), but containing saturated aqueous solutions, as follows:

$\text{Ag} - \text{sat. sol. AgCl} - \text{sat. sol. Ag}_2\text{SO}_4 - \text{Ag}.$

This cell has by no means a zero e. m. f. As is well known it is a concentration cell with respect to the Ag ions. Nor can we assume in this case that all components are in equilibrium, as was the case with cell (II). The two miscible solutions will in fact diffuse into each other.

The same remarks hold good for a cell made up of molten electrolytes, like this type:

$\text{Ag} - \text{molten AgCl} - \text{molten Ag}_2\text{SO}_4 - \text{Ag}.$

The two molten salts are miscible. There is no junction of phases here which could give rise to a potential difference which would compensate for the sum of the potential differences at the

two electrodes. R. Lorenz investigated experimentally cells of this kind and found that they have a considerable e. m. f.

To generalize these results, we may state that:

Every junction of two immiscible phases is the seat of a potential difference. This potential difference is reversible and thermodynamically defined if at least one ion is common to both phases.

For it will always be possible to make up a cell according to the scheme of cell (I). If the common ion is the cation M, a cell may be made up as follows:

metal — phase I containing M' —
phase II containing M' — metal (III).

This system is in a perfect state of equilibrium. Its e. m. f. is zero like the e. m. f. of cell (I). Hence the "phase potential" at the junction of phase I and phase II must be equal and opposite to the difference of the potential differences at the poles.

We also conclude that:

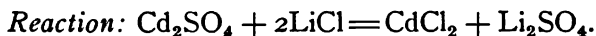
The potential difference at the junction of the two phases does not depend in any way on ionic mobility, like the potential differences between miscible solutions, but they must have properties characteristic of potentials at metallic electrodes.

This similarity with metals is clearly shown by the fact that "phase potentials" may be used to produce an e. m. f. which corresponds to a chemical reaction. For this purpose the "phase potentials" or the phases must be arranged in a certain distinct way, as is done in cell II. The arrangement of cell II may be used to determine, by measuring its e. m. f., the free energy of reaction between solid Ag_2SO_4 and solid NaCl.

Many other arrangements are possible for measuring the free energy of the same reaction. It is not possible to explain here how these different arrangements may be made up. The reaction between Ag_2SO_4 and NaCl is only one example of the large number of reactions the free energy of which can be determined by the measurement of an e. m. f. In the case of many reactions I have carried out the experimental determination, while for many others I have developed the scheme of the arrangement of the experiment.

I wish to give here a few more examples of reactions between

solid salts, the free energy of which was measured by means of similar cells.

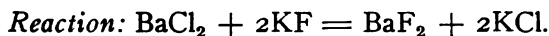


Heat developed: 18,240 cal., corresponding to 76,425 watt-seconds.

E. M. F., calc.: 0.396 volt.

obs.: 0.406 volt at 235° .

0.419 volt at 125° .



E. M. F., calc.: 0.340 volt.

obs.: 0.310 volt at 305° .

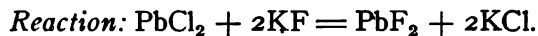
0.315 volt at 302° .



E. M. F., calc.: 0.315 volt.

obs.: 0.300 volt at 260° .

0.304 volt at 210° .



E. M. F., calc.: 0.210 volt.

obs.: 0.231 volt at 310° .

0.220 volt at 210° .

By means of these new galvanic cells reactions of the most different types may be measured; even *i. e.*, such reactions as the formation of a hydrate of a salt from anhydrous salt and water. I have however not been able so far to perform measurements of this last type, though I deem it very well possible. An independent control of a large number of thermochemical data would be possible if the e. m. f. of these new cells was measured, as I have shown in a theoretical paper not yet published.

III. NERNST'S LAW FOR PHASE POTENTIALS.

I now wish to call attention to another property of "phase potentials" in which they resemble potential differences at metals. Nernst has shown that the potential difference between a metal and a solution of one of its salts varies with the concentration of the solution according to a logarithmic law. Exactly the same rule

holds good for "phase potentials" as was also predicted theoretically by Nernst.

Let c_1 be the ionic concentration in phase I of the ion common to both phases, and let c_2 be the ionic concentration in phase II of the same ion, then the "phase-potential" is equal to

$$\frac{RT}{nF} \ln \frac{c_1}{c_2} + \text{const}$$

where R is the constant of the gas law, T the absolute temperature, n the valency, and F the electric charge of a monovalent gram ion.

The proof of this statement is easy. The e. m. f. of cell (III) is zero. The potential difference between the left-hand electrode and phase I is, according to Nernst's theory:

$$\frac{RT}{nF} \ln c_1 + C_1$$

where C_1 is a constant, and the potential difference between the right-hand electrode and phase II is

$$\frac{RT}{nF} \ln c_2 + C_2$$

where C_2 is another constant. The difference of both volumes is the "phase potential" and therefore equals

$$\frac{RT}{nF} \ln \frac{c_1}{c_2} + (C_1 - C_2)$$

Haber first devised a method for directly demonstrating this property of "phase-potentials." The feature of the method is that one of the concentrations c_1 and c_2 is maintained constant, while the other is being varied. *This can be done, if the salt whose concentration is varied is soluble in one phase only.*

To fulfill this condition we use as phase I an aqueous solution of HgNO_3 and as phase II an insoluble salt with the same ion, for instance, HgCl . We then measure the e. m. f. of the following combination:

Calomel electrode — solid HgCl — HgNO_3 aqueous solution — calomel electrode.

If we vary the concentration of the HgNO_3 solution in this cell,

the ionic concentration of the solid HgCl layer will not be affected. Nor will it have any considerable effect on the potential difference between the HgNO_3 solution and the right-hand calomel electrode, since no "phase potential" exists there. Hence the e. m. f. of the whole system must vary with the Hg' concentration according to the logarithmic law, just as in the case of a metallic electrode.

This was shown by experiment to be very exactly true. The concentration of Hg' was changed over a large range by employing NaCl solutions saturated with HgCl (instead of the HgNO_3 solution). As is well-known the Hg' concentration of such a NaCl solution is exceedingly small and yet very accurately defined.

Experiments of the same kind were made with other insoluble salts, like AgCl and CaSO_4 , and their ions in aqueous solution, and the same results were obtained.

Further, Haber and Klemensiewics made experiments on phase potentials of this kind varying the H' concentration. Both phases contained water in their experiments. For instance, if phase I is ice, and phase II is water, then only in phase II acids and bases are soluble, permitting a variation of the H' concentration. For phase I, instead of ice, such materials as glass or solutions of water in organic solvents (like benzol) were taken. Haber developed a complete theory of the change of the potential difference, if the H' concentration is also variable in phase I to a small extent.

IV. PHASE POTENTIALS IN LIVING TISSUES.

The phase potentials are of special importance for electric phenomena in animals and plants. Organic tissues are built up from cells surrounded by membranes. These membranes separate aqueous solutions of various substances (among them salts) from each other. Although the chemical composition is unknown, any membrane may be assumed to have a small⁸ electrolytic conductivity. Then "phase potentials" will exist on both sides of the membrane.

Hence if we cut part of a tissue or take a whole organism and make connection at two points by means of non-polarizable electrodes to a measuring instrument, we will find in general an e. m. f. If we do not find any, we cannot conclude that there are no poten-

⁸ Possibly of the order of a solid salt.

tial differences in the tissue, since it is more likely that potential differences exist, but that they are arranged in such a way as to compensate each other.

The fact is that in many cases a living tissue will give rise to an e. m. f. These bio-electric currents have been studied since Galvani's famous experiment and the scientific literature relating to this subject is enormous. The aim of most of the research work done in this field was to establish relations between electromotive properties of tissues and vital phenomena, but very little has so far been known of the physical nature of these e. m. f.'s.

As to the order of magnitude of these bio-electrical forces, Du Bois-Reymond, who worked all his life in this field, has said that a complete physical explanation was impossible if attempted on the basis of an analogy with cells formed of aqueous solutions. Bio-electric forces resemble much more the e. m. f.'s. of cells with metallic electrodes. This seems strong evidence for the presence of "phase potentials" similar to metal potentials as explained before.

What is the nature of the "phase potentials" which make up the bio-electric forces? With respect to which ions are they reversible? In a recent investigation, Dr. Jacques Loeb and myself succeeded in solving this problem to some extent, and we did so by using the method which I described above. In the case of a layer of HgCl we know we have an electrode reversible with respect to Hg' . With a membrane of unknown composition we cannot tell beforehand for which ion it will be reversible, but we can find it out, in the same way as we demonstrated the Hg' reversibility of HgCl . The membrane is brought into contact with solutions of various salts and in various concentrations and the changes of potential are measured.

The difficulty was to find the right kind of a biological object. Dr. Loeb made the right suggestion by proposing to use parts of plants (fruits and leaves), because of their greater resistance to chemical agents. As a membrane especially, the outer skin of a fruit or leaf gave excellent constant results. The two ends of the leaf were placed in two solutions and from the solutions the current was led to the electrometer with the aid of calomel electrodes. Thus we tried solutions of KCl , NaCl , CaCl_2 , MgCl_2 , BaCl_2 , LiCl

and others, and varied the concentration in each case *on one side*. The most remarkable fact established was that all salts showed the same effect upon the potential of the membrane; with decreasing concentration the solution the concentration of which was changed became more positive, and this occurred very nearly in the amount required by Nernst's formula (the change of e. m. f. at room temperature being 0.058 volt when diluting ten times). The sign of the change shows that the potential is reversible for cations but it is not so for one single cation only, but all cations seem to act in the same way. A phenomenon like this has not yet been known in electrochemistry, but it may be very well explained on the principles given above. What the theory demands for a "phase potential" like this, in order to make it reversible for different cations, is the presence of the slightest amount of all ions in the membrane. For Na, K, Ca this may very well be the case. We can suppose the membrane to contain traces of Na, K, and Ca bound on fatty acids, proteins or carbohydrates in homogeneous mixture. The presence of Li and Ba, however, is not likely, but we can assume a chemical reaction to take place between the outer Li or Ba solution and the Na (or K or Ca) compound of the membrane causing an exchange. In this way we may also get a trace of the ions into the membranes.

The conclusion is to be drawn that salts are insoluble in all membranes for which Nernst's equation holds, as described. For if the salts are soluble in the membrane, an increase of concentration in the outer aqueous solution would also mean an increase of salt concentration in the membrane itself, the concentration ratio of salt in the water to that in the membrane would therefore not increase at all, or increase only slightly; it is this ratio that determines the potential difference according to the formula given above.

Now insolubility means impermeability of the membranes to the salts, as Nernst has shown very clearly. The impermeability of many membranes to salts has also been proved by other methods, such as osmotic experiments. It must, however, be said that Nernst's equation was not always found to hold true; for instance, not in the case of an animal's skin, hence the possibility of permeable membranes cannot be denied.

V. NERNST'S EXCITATION LAW AND THE POTENTIAL DIFFERENCES
UNDER DISCUSSION.

It would seem as though the electrical properties of tissues hardly had much to do with vital processes. This view, however, we may say, is likely to be wrong, although our knowledge of the nature of vital processes is still very incomplete.

As evidence for this statement, I would briefly refer to another electro-physiological phenomenon, namely the effect of alternating current upon movement (or generally speaking excitation) of tissues. It is known that a current changing its intensity can cause excitation, *e. g.*, the movement of a cut-out muscle of a frog. Nernst has shown that a quantitative relation between the intensity and frequency of all alternating currents which are just sufficient to cause the movement of the same muscle can be calculated if it is assumed that the maximum polarization on the junction of membranes is the real cause of the movement.

Now polarization of ordinary metallic electrodes by alternating current is first of all proportional to the intensity of the current, but the polarization also depends on the frequency. The reason is that with the same intensity of current the electric quantity (in coulombs or ampere seconds) which passes for each half period in one direction is, of course, the smaller the higher the frequency. Ordinary diffusion counteracts the changes of concentration. In order to bring about a certain change in the concentration against the forces of diffusion, a certain electric quantity must be passed in one direction, *i. e.*, the period must be above a certain length of time or the frequency must be below a certain limit. A detailed calculation shows that the maximum polarization is inversely proportional to the square root of the frequency. Hence, if we make experiments in which we vary both the intensity and frequency of the alternating current, we will find that the maximum polarization is always the same for all currents for which the quotient of the intensity and the square root of the frequency is constant.

Exactly this same rule holds good for different currents which are just capable of causing movement of the same frog's muscle. This physiological law can claim a considerable accuracy within certain limits. The question how polarization may cause move-

ment is readily answered by analogy with the capillary electrometer. Polarization brings about a change of surface tension on the surface of mercury as well as on the single elements of the muscle. That a change of surface tension may cause a movement in different ways is well known.

Polarization means change of concentration on junctions of phases. If a current passes through a muscle, these changes will take place by electrolytic processes, as is likely from Nernst's theory; but we cannot tell from this theory what is the nature of these changes of concentration. In the experiments with a leaf and an apple, as described, the concentration was artificially changed and the changes of potential thereby determined as to their nature and behavior.

This kind of polarization is by no means confined to plants. Electro-physiological observations by MacDonald⁴ and others, which I cannot discuss here at length, prove very clearly that in the case of animal tissues a change of the concentration of *any* salt also changes the potential on the membranes. Nernst's theory of excitation proves that polarization is the cause of the movement of tissues. Hence we may conclude that with our experiments with leaves and apples we have roughly imitated one of the processes which takes place many thousand times in our nerves, and our muscles, when we move. This is, of course, only one small part of all those complicated physical and chemical processes which, as a whole, are called life.

In conclusion, it may be said that another proof has been obtained for the necessity of salts for vital processes. Dr. Jacques Loeb's experiments on physiological salt actions have furnished abundant material for further progress along these lines.

⁴ MacDonald, Proc. Royal Society, 67, 310.

FERTILIZATION OF THE EGGS OF VARIOUS INVERTEBRATES BY OX-SERUM.

BY JACQUES LOEB AND HARDOLPH WASTENEYS.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

I.

The chemical method of artificial parthenogenesis has thus far been worked out with any degree of completeness, only for the Californian sea urchin, *Strongylocentrotus purpuratus*. In this form it was shown by Loeb that the process of fertilization is composed of two entirely different phases. The one is an alteration or destruction of the surface layer of the egg. This alteration of the cortical layer may or may not result in the formation of a fertilization membrane. The alteration of the surface can be brought about by a great many different means, all of which have a cytolytic effect. The superficial cytolysis starts the development of the egg but leaves the latter with a tendency to perish during the further development. The sickly condition is remedied by a second treatment of the egg, which may consist in putting the eggs for about from 30 to 50 minutes into hypertonic sea water of a certain concentration. If taken out of this solution, the egg develops practically normally.

Experiments on heterogeneous hybridization which Loeb carried out, furnished the evidence that the spermatozoon also causes the development of the egg by carrying two agencies into it, one of which is a cytolytic substance, a lysin, which causes the membrane formation.

Lysins are contained not only in the spermatozoon but in all the cells and in the blood of any animal. Loeb found five years ago that the blood of a worm, *Dendrostoma*, calls forth membrane formation in the unfertilizing egg of the sea urchin. This blood retained its fertilizing power when diluted as much as several hundred times with sea water.

The same author found subsequently that the blood and tissue extract of many animals had the same effect, *e. g.*, the blood of cattle. The fact that the blood of each female does not cause the parthenogenetic development of its own eggs, Loeb explained by the theory, that while the lysins contained in the blood of foreign species can diffuse with comparative ease into the egg and the cells of an animal, the lysins contained in its own blood are prevented from such a diffusion.

It was found impossible to cause the development of the eggs of all female sea urchins by means of foreign blood. This difficulty was overcome by treating the eggs with strontium chloride before they were exposed to the foreign serum. If the sea urchin eggs were put for a short time into a $\frac{3}{8}$ or $\frac{1}{16}$ *M* solution of strontium chloride, a subsequent treatment with ox blood caused them all to form fertilization membranes. When subsequently treated for a short time with hypertonic sea water, most of the eggs developed into normal plutei.

II.

While in this way the mechanism of fertilization was cleared up to a large extent for the sea urchin egg, very little had been accomplished with the eggs of other invertebrates. The eggs of a great many forms had been caused to develop by artificial means but the development was often very abnormal.

Artificial parthenogenesis was caused in the eggs of molluscs by Kostanecki as well as by Loeb, but the development was abnormal in as much as it resulted in the production of larvæ without previous segmentation. In the egg of *Cumingia*, another mollusc, efforts to produce artificial parthenogenesis had failed entirely. In annelids the results were not very satisfactory either. In *Chaetopterus*, *e. g.*, Loeb produced parthenogenetic larvæ, but they developed without segmentation as he first observed and as was later ascertained beyond doubt by F. Lillie.

If the lysin theory of fertilization was correct, it was necessary to find out whether artificial parthenogenesis with an approximately normal type of development can be caused in the eggs of all animals by foreign blood. Experiments which we have carried on this year seem to indicate that this can be done to a large extent.

III.

We first ascertained that the eggs of *Arbacia* behave essentially like those of *Strongylocentrotus purpuratus*. The eggs of *Arbacia* are sensitized by putting them for a short time into a $\frac{1}{2}$ M solution of strontium chloride. They were then exposed for about 10 minutes to ox-serum which had been rendered isotonic with sea water through the addition of sodium chloride. The eggs were then transferred for 20 minutes into hypertonic sea water. Such eggs developed normally into plutei. The only difference between the behavior of the eggs of *Arbacia* and *Strongylocentrotus* is that the eggs of *Arbacia* do not form a very distinct membrane. It is needless to say that the necessary controls were made and that we made sure that the treatment of the eggs with strontium chloride or with strontium chloride and subsequently with the hypertonic solution, did not lead to the formation of embryos, although occasionally a few segmentations could be brought about in this way.

We next worked with the eggs of *Cumingia* which had been found to be refractory to the other methods of artificial parthenogenesis. We obtained an apparently perfectly normal segmentation of the eggs and the formation of larvæ, by treating them in the following way: The eggs were sensitized to the effects of serum by placing them for from 2 to 4 minutes into a $\frac{1}{2}$ M solution of strontium chloride. They were then placed for five minutes into ox-serum rendered isotonic with sea water and diluted with an equal part of a $M/2$ solution of $\text{NaCl} + \text{CaCl}_2 + \text{KCl}$. After having been freed from all traces of serum by repeated washing in a Ringer solution they were transferred for 60 minutes into hypertonic sea water (50 c.c. sea water + 8 c.c. $2\frac{1}{2}$ M NaCl). Control experiments showed that the treatment with serum is the essential factor in this process.

We induced segmentation in the eggs of *Chætopterus* by putting them for from $1\frac{1}{2}$ to $2\frac{1}{2}$ minutes into a mixture of 25 c.c. $\frac{1}{2}$ M strontium chloride + 25 c.c. $M/2$ $\text{NaCl} + \text{CaCl}_2 + \text{KCl}$, then for ten minutes into ox-serum diluted with its own volume of the above mentioned solution and then by putting them for thirty minutes into hypertonic sea water. From fixed and stained prepa-

rations which Dr. Bancroft made for us, we made sure that the nuclear and cell division was real and not merely apparent.

While the method needs to be perfected in some details, the experiments show that it is possible to induce, with the aid of foreign blood serum, parthenogenetic segmentation and development into larvæ, in eggs which had been found refractory to the other methods of artificial parthenogenesis. The lysin theory of fertilization is therefore more generally applicable.

DIE URSACHEN DES VERLETZUNGSSTROMES.*

VON JACQUES LOEB UND REINHARD BEUTNER.

(Aus den Laboratorien des Rockefeller Institute for Medical Research, New York.)

Mit 13 Figuren im Text.

I.

Wenn wir an einer Stelle eines Apfels die Rinde beseitigen und von der verletzten und einer unverletzten Stelle der Rinde mit identischen Salzlösungen ableiten, so verhält sich, wie zu erwarten, die verletzte Stelle negativ zur unverletzten. Die EMK beträgt 20 bis 100 Millivolt. Wir wiesen in der vorausgehenden Arbeit¹⁾ auf die Schwierigkeit bei der Erklärung dieser EMK hin. Tatsache ist, dass die Verdünnung der Ableitungsflüssigkeit auf einer Seite dieselbe positiver macht. Nun finden wir aber, dass selbst bei Ableitung mit einer grammolekularen Lösung eines Salzes die verletzte Seite noch negativ ist. Das würde voraussetzen, dass die Konzentration des Apfelsaftes eine Grösse besitzen müsste, die den Tatsachen nicht entspricht. Wir bestimmten die Leitfähigkeit des ausgepressten Saftes eines Apfels und erhielten $K_{18^\circ} = 0,002,26$. Daraus folgt, dass der Gehalt dieses Saftes an dissoziierten Elektrolyten einer Konzentration von N/58 entspricht, wenn es sich um KCl handelt, von N/170, wenn es sich um HCl handelt.

Die folgenden Versuche hatten den Zweck, die Ursache des Verletzungsstromes beim Apfel zu ermitteln.

Der Umstand, dass der Apfelsaft Säure (Apfelsäure) enthält, veranlasste uns zuzusehen, ob beim unverletzten Apfel eine Potentialdifferenz auftritt, wenn wir an einer Stelle der unverletzten Rinde einmal mit einer Salzlösung, das andere Mal mit einer Säurelösung der gleichen Konzentration ableiten. Diese Versuche ergaben das Resultat, dass wir auf diese Weise EMKe von der Grossenordnung

* Eingegangen am 11. Juli 1912.

1) Diese Zeitschr., 41, 1, 1912.

des Verletzungsstromes erhalten. Es sei nochmals ausdrücklich betont, dass in diesen Fällen die Ableitung mit Säure sowohl wie mit Salzen von der unverletzten Oberfläche der Rinde des Apfels erfolgte.

Wir geben eine Reihe von Beispielen:

Kette: $\frac{n}{10}$ -KCl | Apfel | $\frac{n}{2500}$ -KCl EMK 0,115 Volt
 $\frac{n}{10}$ -KCl | Apfel | $\frac{n}{2500}$ -KCl EMK 0,050 "

Die Differenz beträgt 0,065 Volt.

Eine Wiederholung des Versuches an einem zweiten Apfel ergab für $\frac{n}{2500}$ -KCl 0,120 Volt, für $\frac{n}{2500}$ -HCl 0,062 Volt. Differenz 0,058 Volt. Dann wurde die Rinde des Apfels an einer Stelle beseitigt und mit $\frac{n}{2500}$ -KCl an dieser und einer unverletzten Stelle der Rinde abgeleitet. Der Verletzungsstrom betrug 0,087 bis 0,108 Volt.

Dann wurden Versuche mit höheren Konzentrationen angestellt, wobei aber stets identische Konzentrationen von KCl und HCl zum Vergleich kamen. Je drei Versuche wurden immer angestellt. Ableitung von zwei unverletzten Stellen der Oberfläche.

$\frac{n}{10}$ -KCl Apfel			
$\frac{n}{500}$ -KCl; EMK.... (I) 0,090	(II) 0,092	(III) 0,094	Volt
$\frac{n}{10}$ -KCl Apfel			
$\frac{n}{500}$ -HCl; EMK.... (I) 0,032	(II) 0,040	(III) 0,043	Volt
Differenz	<u>0,058</u>	<u>0,052</u>	<u>0,051</u> Volt

Der Verletzungsstrom war bei Ableitung mit $\frac{n}{500}$ -KCl beiderseits, d. h. von der verletzten und unverletzten Stelle, 0,033 bis 0,103 Volt.

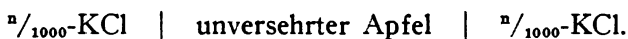
Bei der nächsten Versuchsreihe wurden $\frac{n}{100}$ -Lösungen zur Ableitung verwendet; beide Ableitungen fanden immer von unverletzten Stellen der Rinde statt.

$\frac{n}{10}$ -KCl Apfel			
$\frac{n}{100}$ -KCl; EMK.... (I) 0,054	(II) 0,056	(III) 0,056	Volt
$\frac{n}{10}$ -KCl Apfel			
$\frac{n}{100}$ -HCl; EMK.... (I) <u>0,021</u>	(II) <u>0,034</u>	(III) <u>0,038</u>	Volt
Differenz	<u>0,033</u>	<u>0,022</u>	<u>0,018</u> Volt

Der Verletzungsstrom war bei Ableitung mit $\frac{n}{100}$ -KCl beiderseits

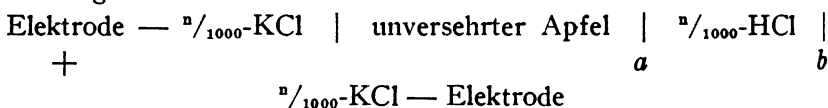
0,035 bis 0,044 Volt, mit $\frac{n}{100}$ -HCl beiderseits 0,011 bis 0,102 Volt. 1)

Man sieht hieraus schon, dass bei Ableitung mit einer Salzlösung und einer Säurelösung von gleicher Konzentration von der unversehrten Rinde des Apfels EMKe entstehen, die von nahezu derselben Grössenordnung, jedoch stets etwas kleiner sind wie die beim Verletzungsstrom gefundenen, und in derselben Richtung wirken, wenn man annimmt, dass die Säure auf der Innenseite der Apfelschale sich befindet. Die Übereinstimmung wird aber eine praktisch vollständige, wenn wir berücksichtigen, dass die Negativität bei Säureableitung durch ein Diffusionspotential vergrössert wird. Gemessen wurde



In den Elektroden befand sich ebenfalls $\frac{n}{1000}$ -KCl. Es war eine kleine EMK von 0,007 Volt vorhanden, in dem Sinne, dass rechts + war, infolge einer ungleichmässigen Beschaffenheit des Apfels.

Darauf wurde rechts mit $\frac{n}{1000}$ -HCl abgeleitet, die Kette war also wie folgt:



EMK: —0,025 Volt, links negativ. Die Gesamtänderung betrug also 0,032 Volt, Säure negativ. In der Kette ist links +, rechts —. Durch das Diffusionspotential wird die Säure negativ gegen das Salz bei *b* geladen; da es im entgegengesetzten Sinne wirkt wie die Gesamtkraft der obigen Kette, so muss der Wert des Diffusionspotentials, der in diesem Falle 0,027 Volt betrug, zur beobachteten Potentialdifferenz addiert werden. Die wirkliche Änderung der Potentialdifferenz bei *a* beim Ersatz der $\frac{n}{1000}$ -KCl durch $\frac{n}{1000}$ -HCl betrug also $0,032 + 0,027 = 0,059$ Volt. Um die wahren Werte der Änderung der Potentialdifferenz beim Ersatz von Salzlösung durch Säurelösung zu erhalten, müssen wir immer den nicht unbeträchtlichen Wert des Diffusionspotentials zu dem beobachteten Wert hinzuaddieren. Die Berechnung des Diffusionspotentials ist schwierig, wenn Säure und Salzlösung verschie-

1) Auf Seite 17 unserer früheren Arbeit haben wir bereits einen ähnlichen Versuch wie die hier beschriebenen erwähnt.

dener Konzentration sich berühren, wie es bei den meisten unserer Versuche der Fall ist, und unterbleibt deshalb hier. Das Diffusionspotential dürfte stets ca. 20 bis 30 Millivolt betragen und wirkt stets in dem gleichen Sinn. Berücksichtigen wir das, so dürfen wir sagen, dass bei Ableitung mit einer Säurelösung und einer gleich konzentrierten Salzlösung an der unversehrten Rinde des Apfels EMKe entstehen, die dem Sinne und der Grössenordnung nach den beim Verletzungsstrom beobachteten gleich sind.

II. DIE GLEICHE WIRKUNG ISOHYDRISCHER SÄUREN.

Wir versuchten festzustellen, ob für die elektromotorische Wirkung der Säuren die Konzentration der Wasserstoffionen in Betracht kommt, und verglichen die Wirkung isohydrischer Säuren.

I	{	$\frac{n}{100}$ -Essigsäure	0,052 Volt
		$\frac{n}{2500}$ -HCl	0,050 "
II	{	$\frac{n}{2500}$ -Essigsäure	0,087 Volt
		$\frac{85}{10000}$ -n-HCl	0,083 "
III	{	$\frac{n}{500}$ -HCl	0,035 Volt
		$\frac{n}{4}$ -Essigsäure	0,048 "
		$\frac{n}{500}$ -HCl	0,043 "
		$\frac{n}{500}$ -HCl	0,040 "

Der Wert für Essigsäure in Versuch III ist ein Anfangswert. Lässt man in so konzentrierter Essigsäure den Apfel längere Zeit liegen, so steigt die EMK allmählich, was durch das Eindringen der Essigsäure zu erklären ist. Konzentrierte HCl verändert bei längerer Einwirkung die EMK ebenfalls, jedoch in umgekehrtem Sinne.

IV	{	$\frac{n}{500}$ -Trichloressigsäure	0,002 Volt
		$\frac{n}{500}$ -HCl	0,004 "
V	{	$\frac{n}{100}$ -Trichloressigsäure	— 0,002 Volt
		$\frac{n}{100}$ -HCl	— 0,004 "

Das erweckt den Anschein, als ob die Konzentration der Wasserstoffionen für die Wirkung der Säure massgebend sei.

III. DIE WIRKUNG DES PRESSSAFTES DES APFELS.

Wir erwarteten, dass der Presssaft des Apfels sich wie eine Säurelösung verhalten würde. Statt dessen fanden wir, dass derselbe

sich wie eine KCl-Lösung von nahezu gleicher Leitfähigkeit verhält. Einige Beispiele sollen das erläutern.

$\frac{n}{50}$ -KCl		Apfel		Presssaft	EMK 0,047 Volt
		intakt		verletzt	
$\frac{n}{50}$ -KCl		Apfel		$\frac{n}{50}$ -KCl	EMK 0,044 Volt
		intakt		verletzt	

In diesem Beispiel war der Saft an der verletzten Stelle angebracht. Im folgenden Versuch wurden Presssaft und KCl an unverletzter Stelle angebracht.

$\frac{n}{50}$ -KCl		Apfel		Presssaft	EMK 0,012 Volt
		verletzt		intakt	
$\frac{n}{50}$ -KCl		Apfel		$\frac{n}{50}$ -KCl	EMK 0,012 Volt
		verletzt		intakt	

Der Presssaft wirkt also nicht wie eine Säurelösung, sondern wie eine Salzlösung von gleicher Leitfähigkeit. Diese Tatsache wurde von uns zuerst als eine neue Schwierigkeit empfunden, da ja im allgemeinen angenommen wird, dass die Ursache des Verletzungsstroms an der verletzten Oberfläche zu suchen ist. Würde nun an der verletzten Oberfläche Säure gebildet, so wäre der Verletzungsstrom ohne weiteres auf Grund der im vorigen Abschnitt mitgeteilten Versuche erklärt. Auf der Schnittfläche wird aber nicht Säure gebildet, sondern Presssaft, nämlich der Saft, der durch das Durchschneiden der Zelle und Gewebe des Apfels ausfließt. Derartiger Saft kann aber, wie wir eben sahen, nicht die Ursache des Ruhestromes sein.

IV. WIRKUNG EINER QUETSCHUNG DER OBERFLÄCHE DES APFELS.

Wenn wir mit dem Finger auf die Oberfläche des Apfels drücken, so kommt es infolge der Quetschung zu einem Erguss des Presssaftes unter der gedrückten Rinde. Wenn wir nun mit identischen Salzlösungen von der gequetschten und einer nicht gequetschten Stelle der Rinde ableiten, so finden wir, dass die gequetschte Stelle sich negativ zu der nicht gequetschten Stelle verhält. So fanden wir in einem Falle bei Ableitung von zwei unversehrten Stellen der Oberfläche des Apfels mit identischen Salzlösungen eine EMK von 0,001 Volt. Dann wurde die eine Ableitungsstelle mit dem Finger gedrückt, so dass eine bleibende geringe Deformation eintrat; während die andere Ableitungsstelle intakt blieb. Nun ergab sich bei

derselben Ableitung eine EMK von 0,044 Volt im entgegen-gesetzten Sinne. Die Gesamtänderung war also 0,045 Volt und die gequetschte Stelle negativ. Ein zweiter Versuch verlief wie folgt:

Unversehrter Apfel 0,008 Volt
 An einer Ableitungsstelle gequetscht 0,058 "
 Nach 14 Minuten 0,048 "

Gesamtänderung 0,066 resp. 0,056 Volt, wobei die gequetschte Stelle negativ war. Die Quetschung wirkt also dem Sinne nach wie eine Verletzung oder Entfernung der Rinde, auch die Grössenordnung der Änderung der EMK ist die gleiche.

Das elektromotorische Verhalten der Rinde wird durch diese Methode der Quetschung nicht geändert. Das lässt sich durch folgende Versuche beweisen. Wir haben in der vorausgehenden Abhandlung gezeigt, dass dieselbe Änderung der Konzentration der Ableitungsflüssigkeit auf der unverletzten Rinde des Apfels einen mehr als doppelt so grossen Unterschied der EMK bedingt als auf der verletzten Seite. Untersuchen wir nun den Einfluss der Konzentrationsänderung auf der gequetschten Seite des Apfels, so finden wir, dass dieselbe wie beim völlig unverletzten Apfel verläuft. Folgendes Beispiel möge genügen: Die Ableitungsflüssigkeit an der nicht gequetschten Stelle blieb konstant, die an der gedrückten Stelle wurde variiert.

Konzentration an der gequetschten Stelle	EMK	Differenz
$\frac{n}{10}$ -KCl	- 0,048 Volt	0,048 Volt
$\frac{n}{100}$ -KCl	$\pm 0,0$ "	0,048 "
$\frac{n}{1000}$ -KCl	+ 0,048 "	0,046 "
$\frac{n}{100}$ -KCl	+ 0,002 "	

Wie man sieht, sind diese Differenzen dieselben, die wir in der vorausgehenden Abhandlung für dieselben Konzentrationsänderungen an der normalen Rinde fanden.

Im normalen Apfel sind normale Gewebe und Zellen in Berührung mit der Rinde. An der gequetschten Stelle tritt Presssaft aus und tritt an die Stelle der normalen Berührungssubstanzen. Nehmen wir an, dass bei normaler Struktur des Apfels eine Lamelle einer Säurelösung (oder eines elektromotorisch wie Säure wirkenden Stoffes) an der inneren Oberfläche der Rinde vorhanden ist, und dass bei der Quetschung diese Lamelle durch den Presssaft ersetzt

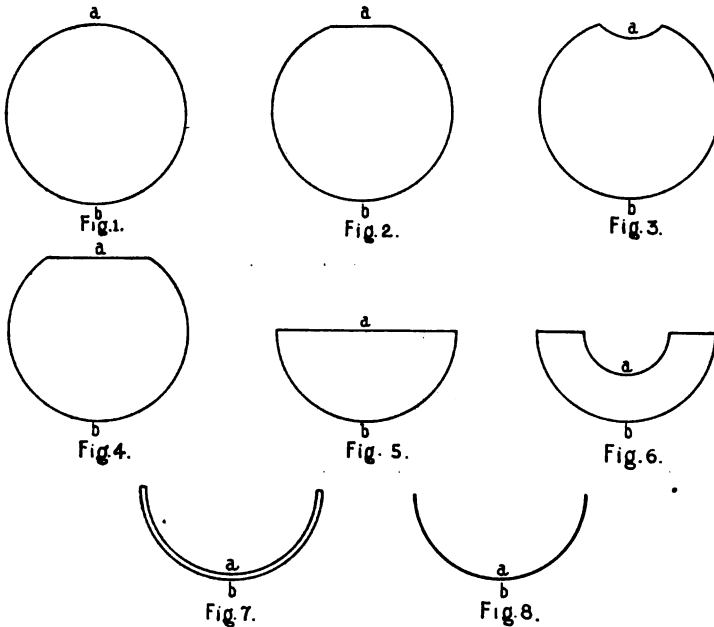
wird, so finden der Verletzungsstrom und alle anderen bisher besprochenen Erscheinungen ihre Erklärung.

Die wesentliche Potentialdifferenz hat nach dieser Annahme ihren Sitz an der inneren Grenze von Rinde und Apfelsubstanz. Leiten wir von zwei intakten Oberflächenelementen ab, so heben sich diese Potentialdifferenzen wegen ihres entgegengesetzten Zeichens auf. Entfernen wir die Rinde oder ihre Epidermis an einer Stelle, so bleibt nur die Potentialdifferenz an der inneren Oberfläche der intakten Rinde übrig und diese lässt die verletzte Stelle negativ erscheinen. Ersetzen wir an einer Seite unter der Rinde die natürliche Säurelösung oder wie eine Säure wirkende Substanz durch den wie eine Salzlösung wirkenden Presssaft, so bleibt nur der Potentialsprung an der Innenfläche der nicht gequetschten Rinde übrig und dieser lässt die gequetschte Seite negativ erscheinen. Wir haben hier nur von einer natürlicherweise an der Innenseite der Rinde bestehenden Säureschicht oder einer elektromotorisch wie Säure wirkenden Substanz gesprochen. Das ist nur der Kürze halber geschehen, solche Schichten könnten ebenso gut auch an der Grenze vieler Zellen im Innern namentlich in der Nähe der Rinde angenommen werden. Dieser Versuch widerlegt auch die Annahme, als ob an der Verletzungsstelle Säure gebildet würde. Wenn das der Fall wäre, so sollte ja die gequetschte Stelle positiver werden, denn Ersatz von Salz durch Säure müsste auf der Innenseite im entgegengesetzten Sinne wie auf der Aussenseite wirken, d. h. positivierend.

V. MESSUNG VON VERLETZUNGSSTRÖMEN BEIM APFEL MIT FORTSCHREITENDER AUSHÖHLUNG. EINFLUSS DER ENTFERNUNG DER VERLETZUNGSSTELLE VON DER RINDE.

Bei der Messung des Verletzungsstromes verfahren wir folgendermassen: Die untere unverletzte Stelle *b* des Apfels lag in einer Schale, die mit der Ableitungsflüssigkeit gefüllt war. An der oberen entgegengesetzten Stelle *a* (Fig. 1 bis 8) wurde erst eine flache Verletzung angebracht, in welche die andere Ableitungsflüssigkeit gebracht wurde, die mit der unteren Flüssigkeit in diesem Versuch identisch war, nämlich $\frac{1}{50}$ -KCl. Dann wurde immer mehr von dem Apfel von oben angefangen abgetragen, wodurch die Verletzungsstelle immer näher an die untere unverletzte Rinde rückte. Dabei zeigte sich ein sehr charakteristisches Verhalten.

Anfangs war die Annäherung der Verletzungsstelle an die untere unverletzte Rinde wirkungslos. Zuletzt aber, als die Schnittfläche der unteren Rinde nahe kam, fing die EMK an rasch zu sinken und wurde alsbald Null. Folgendes Beispiel diene zur Illustration.



- Fig. 1. Unverletzter Apfel 0,001 Volt
 Fig. 2. Oben kreisförmiges Stück der Epidermis
 mit ca. $\frac{1}{2}$ cm. Radius entfernt 0,041 Volt
 Fig. 3. In die verletzte Stelle ein 1 cm tiefes Loch
 gemacht 0,037 Volt
 Fig. 4. Unterhalb des Loches ein $1\frac{1}{2}$ cm dickes
 Segment des Apfels abgetragen und
 von der neuen oberen Schichtfläche
 abgeleitet 0,040 Volt
 Fig. 5. Neuer Schnitt durch das Zentrum des
 Apfels und die obere Hälfte des Apfels
 entfernt und von der neuen oberen
 Schnittfläche abgeleitet 0,039 Volt
 Nach 6 Minuten 0,040 Volt

- Fig. 6. Eine weitere Schicht des Apfels von oben abgetragen, und Mark konzentrisch mit der unteren Rinde entfernt 0,040 Volt
 Nach 9 Minuten 0,038 Volt
- Fig. 7. Weitere Schicht konzentrisch mit Rinde abgetragen, so dass die obere Schnittfläche nur $\frac{1}{4}$ cm. über der Rinde liegt... 0,020 Volt
 Nach 8 Minuten 0,020 Volt
- Fig. 8. Reste des Markes entfernt, so dass eine Kugelschale übrig ist, die wesentlich nur aus Rinde besteht 0,008 Volt
 Nach 4 Minuten 0,009 Volt
 Nach 11 Minuten 0,011 Volt

Dieser Versuch wurde mit wesentlich demselben Resultat wiederholt. Es ergab sich stets, dass erst, wenn die Schnittfläche der unverletzten Rinde, von der abgeleitet wurde, so nahe kam, dass die Ableitungsflüssigkeit sich mit der natürlichen Flüssigkeit an der inneren Fläche der Rinde mischen musste (Fig. 7 und 8), eine Änderung der EMK bemerkbar wurde, und zwar in dem Sinne, dass dieselbe abnahm. Je vollständiger der Ersatz der natürlichen Flüssigkeit an der inneren Grenze von Mark und Rinde durch die zur Ableitung dienende Salzlösung ist, um so mehr sinkt der Verletzungsstrom, der schliesslich meist ganz verschwindet, wenn die Salzlösung die innere Seite der Rinde ganz berührt.

Die Aussenfläche der Rinde ist dabei elektromotorisch unverändert. Prüft man den Einfluss der Konzentrationsänderung an der Aussenfläche einer Schale die vom Mark sorgfältig und schonend, d. h. ohne Risse zu verursachen, befreit ist, so findet man, dass die Aussenfläche einer so isolierten Rinde sich genau wie die Rinde des unversehrten Apfels verhält, wie das folgende Beispiel zeigt:

Konzentration der äusseren Lösung	EMK	Differenz
$\frac{n}{10}$ -KCl	0,015 Volt	
$\frac{n}{80}$ -KCl	0,046 „ >	0,031 Volt
$\frac{n}{250}$ -KCl	0,080 „ >	0,034 „
$\frac{n}{1350}$ -KCl	0,111 „ >	0,032 „
$\frac{n}{10}$ -KCl	0,011 „	

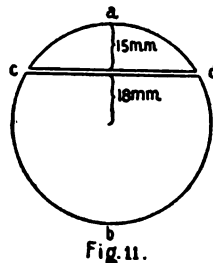
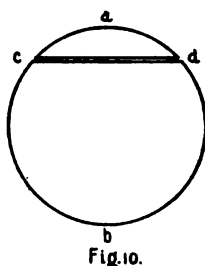
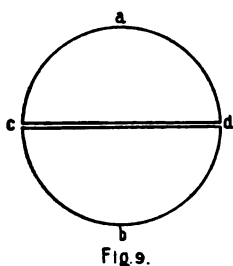
Ein bis zur Rinde ausgehöhlter Apfel wurde innen und aussen mit $\frac{n}{10}$ -KCl abgeleitet; Verletzungsstrom 0,015 Volt, nach 5 Minuten

0,014 Volt. Die äussere Lösung wurde alsdann durch verdünntere ersetzt.

Diese Werte stimmen mit denen, die am unversehrten Apfel bei denselben Konzentrationsänderungen beobachtet werden, überein.

VI. EINFLUSS VON SCHNITTEN QUER DURCH DEN APFEL AUF DIE EMK BEI ZWEI ENTGEGENGESETZTEN UNVERLETZTEN STELLEN.

Nach dem Gesagten ist folgender Versuch leicht zu verstehen. Ein Apfel wird halbiert und die Stücke werden mit den Schnittflächen *c, d* (Fig. 9) wieder aufeinander gelegt. Die Schnittebene liegt senkrecht zu der Verbindungslinie der zwei Ableitungsstellen *a* und *b*. Wie nach der Symmetrie der Anordnung zu erwarten war,



trat keine Änderung der EMK ein. Durchschneiden wir einen Apfel senkrecht zur Verbindungslinie der beiden Ableitungsstellen, aber so dass der Schnitt *cd* nicht durch die Mitte der Linie geht, sondern der einen Ableitungsstelle näher liegt als der anderen (Fig. 10), so tritt folgendes ein. Sobald die Schnittfläche nicht zu nahe an die eine Ableitungsstelle gerückt wird, findet keine oder nur eine geringe Änderung der EMK statt, trotz der Asymmetrie der Lage der Schnittfläche. Erst wenn die Schnittfläche sehr nahe an die eine Ableitungsstelle, z. B. *a*, heranrückt, tritt eine merkliche Änderung ein.

Bei einem unverletzten Apfel wurde die höchste und niederste Stelle wie in der beschriebenen Anordnung durch eine Salzlösung derselben Konzentration ($\frac{1}{10}$ -KCl) abgeleitet und eine EMK von —0,008 Volt gefunden. Nach 6 Minuten—0,006 Volt. Dann wurde der Apfel 18 mm über dem Zentrum und 15 mm unter der höchsten Kuppe der Rinde senkrecht zur Verbindungslinie der beiden Ableitungsstellen *a* und *b* (Fig. 11) durchschnitten, die beiden

Stücke mit der gemeinsamen Schnittfläche wieder aufeinander gelegt und wieder wie vorhin von der intakten Rinde bei *a* und *b* abgeleitet. EMK — 0,010, nach 3 Minuten — 0,007 Volt, also unverändert.

In einem andern Versuche wurde der Schnitt 10 mm unter der Kuppe des Apfels angelegt und auch hier bewirkte die Durchschneidung, trotzdem die Schnittfläche der einen Ableitungsstelle so viel höher lag als der anderen, keine Änderung der EMK. Lag der Schnitt aber der oberen Kuppe ganz nahe, so trat eine merkliche Änderung der EMK im Vergleich mit dem unverletzten Apfel ein, wie folgende Beispiele zeigen. Am unverletzten Apfel wurde die EMK 0,001 Volt gefunden. Dann wurde senkrecht zur Verbindungslinie der beiden Ableitungsstellen, aber 6 mm unter der oberen Kuppe, durchgeschnitten und die Stücke wieder mit der gemeinsamen Schnittfläche aufeinander gelegt. Die EMK war nunmehr bei derselben Ableitung 0,038 Volt, nach 4 Minuten 0,037 Volt. Die der Schnittfläche nähere Ableitungsstelle war negativ. In einem andern Falle lag der Schnitt nur 1 mm unter der oberen Kuppe. Die Änderung der EMK nach der Durchschneidung und nachdem die Stücke wieder aufeinander gelegt waren, betrug 0,036 Volt. Also erst wenn die Schnittfläche einer Ableitungsstelle so nahe liegt, dass ein teilweiser Ersatz der natürlichen Flüssigkeit an der innern Oberfläche der Rinde oder in der Nähe dieser Oberfläche durch Presssaft stattfinden kann, tritt eine Änderung der EMK ein; und zwar in dem Sinne, als ob eine Säure durch Presssaft ersetzt würde.

Viele Physiologen stellen sich vor, dass das absterbende Gewebe negativ ist gegen das lebende. Das Bedenkliche an dieser Ausdrucksweise ist ihre Unbestimmtheit, die dieselbe jeder physikalisch-chemischen Prüfung unzugänglich macht. Gelegentlich wird angenommen, dass das Absterben in einer Säuerung besteht, die von der Schnittfläche ausgehend nach und nach sich in immer tiefer gehende Regionen ausbreitet. Unsere Versuche zeigen, dass diese Annahme hier nicht ohne sehr gekünstelte Hilfsannahmen möglich ist. Wenn wir nämlich ein kleines Segment des Apfels abschneiden, dasselbe wieder in seiner alten Orientierung auf den Rest des Apfels auflegen und dann von der unverletzten Rinde dieses Segments und vom entgegengesetzten Ende des Apfels ableiten (wie in Fig. 10), dann erweist sich die der Schnittfläche näher liegende Ableitungs-

stelle als negativ gegen die ferner liegende. Hätte die Schnittfläche eine Säuerung des nahe gelegenen Gewebes zur Folge, so sollte auf der Innenseite der Rinde des kleineren Stückes eine stärkere Säuerung vorhanden sein, als auf der Innenseite des von der Schnittfläche weit entfernten entgegengesetzten Endes. Das würde aber bedingen, dass die der Schnittfläche näher liegende Rinde erheblich positiver sein müsste als die ferner liegende.

Wenn man aber mit dem Wort „Absterben“ die Vorstellung verbinden wollte, dass dasselbe in einer Diffusion des Zellinhaltes oder Presssaftes aus den Zellen bestehe, so würde das unseren Versuchen und Schlüssen nicht widersprechen. Bedenklich wäre nur, wie schon erwähnt, der unbestimmte Charakter der Ausdrucksweise.

VII. OBERFLÄCHLICHE VERLETZUNG AUSSER KONTAKT MIT EINER ABLEITUNG.

Es stimmt mit dem Gesagten auch überein, dass eine Verletzung in der Nähe einer Ableitungsstelle nur dann die EMK ändert, wenn der durch die Verletzung frei gemachte Presssaft die andere Innenseite der Rinde an der Ableitungsstelle erreichen kann. Entfernt man die Rinde unterhalb der Ableitungsstelle in einem ringförmigen Bezirk, so hat das keinen oder kaum einen Einfluss auf die EMK.

In einem Versuche wurde bei einem unverletzten Apfel bei *a* und *b* (Fig. 12) abgeleitet; EMK $-0,012$ Volt. Dann wurde ein Einschnitt in die Rinde längs *CD* gemacht; EMK $-0,012$ Volt.

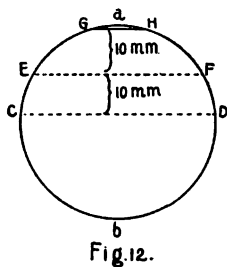


Fig. 12.

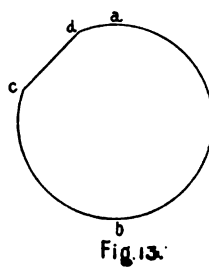


Fig. 13.

Dann wurde die Rinde oberhalb *CD* in einen 10 mm weiten Ring abgeschält bis zur Linie *EF*. EMK $-0,008$ Volt. Dann wurde ein neues, 10 mm breites Stück Rinde *EFGH* ringförmig abgeschält, so dass nur noch ein Apfelsegment an der Kuppe von $1\frac{1}{2}\text{ mm}$ Höhe mit Rinde bedeckt war. EMK $+0,008$ Volt.

Dann wurde dieses kleine Segment durch einen Schnitt senkrecht zur Verbindungslinie *ab* ganz abgetrennt, wieder in der alten Orientierung auf den Apfel gelegt; EMK 0,039 Volt. Man sieht also, dass die letztere kleine Verletzung einen ungleich viel grösseren Einfluss auf die EMK hatte wie die ausgedehnte Verletzung, die die Abtrennung der Rinde in der Nähe des Segments bewirkt hatte. Andere Versuche dieser Art gaben ganz ähnliche Resultate.

Es wurde dann versucht, ob eine Abtrennung eines Stückes von Rinde und Mark in der Nähe einer Ableitungsstelle wie in Fig. 13 einen Erfolg hätte. Das war, wie wir erwarteten, nicht der Fall. Vorher bei Ableitung von *a* und *b* EMK — 0,006 Volt. Dann wurde ein Stück *cd* abgeschnitten, EMK — 0,001 Volt.

Da bei dieser Verletzung keine nennenswerte Diffusion des Presssaftes von der Wunde an die innere Oberfläche der Rinde der Ableitungsstelle stattfinden kann, so tritt auch keine nennenswerte Änderung der EMK ein.

VIII. THEORETISCHE BEMERKUNG.

Durch die vorstehenden Überlegungen und Experimente ist es wahrscheinlich gemacht, dass der Verletzungsstrom physikalisch-chemisch durch eine Kette vom Typus

Säurelösung | Apfelschale | Salzlösung I

wo Säure- und Salzlösung gleiche Konzentration aufweisen, hervorgerufen wird.

Eine solche Kette kann nicht als Konzentrationselement gedeutet werden. Ein Konzentrationselement wäre z. B. die Kette

Salzlösung | Apfelschale | Salzlösung II
konz. c_1 | | konz. c_2

Die Schwierigkeiten, die bei der Erklärung des Verletzungsstromes auf dieser Grundlage entstehen, wurden eingangs genannt.

Die Ursache der EMK in Kette II ist ein einfacherer Konzentrationsausgleich der beiden Salzlösungen, bei Kette I aber ist die Ursache eine chemische Reaktion, und zwar in der Schale. Im Sinne der früher von Loeb ausgesprochenen Ansicht, dass Salz-, Eiweiss- oder Fettverbindungen sich durch Umsatz im Gewebe bilden, wäre diese Reaktion etwa



wo X das Anion der Fettsäure oder des Proteins bedeutet.

ZUSAMMENFASSUNG.

1. Leitet man an einer Stelle der unverletzten Oberfläche des Apfels mit einer Salzlösung, an einer zweiten mit einer gleich konzentrierten Säurelösung ab, so beobachtet man eine EMK, die von derselben Grössenordnung ist wie der Verletzungsstrom beim Apfel. Die Stelle, die mit der Säure in Berührung ist, ist negativ zur Stelle, die mit der Salzlösung in Berührung ist.

2. Isohydriche Säuren wirken gleich stark.

3. Der Presssaft des Apfels wirkt nicht wie eine Säurelösung, sondern wie eine Salzlösung von derselben Leitfähigkeit.

4. Übt man einen Druck auf die unverletzte Oberfläche des Apfels, so findet ein Erguss von Presssaft unter der Rinde des Apfels statt, und zugleich beobachtet man bei Ableitung von der gedrückten und einer unversehrten Stelle der Rinde eine EMK von der Grössenordnung und demselben Zeichen, als ob an der gedrückten Stelle die Rinde entfernt wäre. Es lässt sich aber zeigen, dass die Rinde bei der Quetschung keine Änderung ihrer elektromotorischen Eigenschaften erleidet.

5. Aus diesen und den vorausgehenden Tatsachen sind wir geneigt zu schliessen, dass im unverletzten Apfel an der Innenschicht der Rinde und der benachbarten Zellen eine Schicht einer Lösung besteht, die Säure enthält, oder eine Substanz, die elektromotorisch wie eine Säure wirkt. Durch die Quetschung wird diese Schicht durch Presssaft ersetzt und damit die normalerweise an dieser Stelle bestehende Negativität beseitigt.

6. Messungen von Verletzungsströmen des Apfels bei verschieden tiefer Aushöhlung bestätigen diese Annahme: der elektromotorisch unwirksame Presssaft oder die ableitende Salzlösung verdrängen die wirksame innere Rindenschicht bei vollständiger Aushöhlung und dadurch sinkt die EMK.

7. Aus unseren Versuchen folgt, dass beim Apfel der Verletzungsstrom kein Konzentrationsstrom ist, sondern durch eine chemische Reaktion bestimmt ist.

THE DIRECT TREATMENT OF SYPHILITIC DISEASES OF THE CENTRAL NERVOUS SYSTEM.

A PRELIMINARY COMMUNICATION.

BY HOMER F. SWIFT, M.D., AND ARTHUR W. M. ELLIS, M.B.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

The involvement of the central nervous system in lues is one of the most important phases of the disease. This involvement is not only of interest in the tertiary and parasymphilitic periods, but is also one of the striking features of the early stages. It is the object of this communication to present a preliminary report on the direct local treatment of syphilis of the nervous system.

Since the etiologiical rôle of *Treponema pallidum* has been established, many mooted points in the life history of the disease have been elucidated. The early general distribution of the virus is well known. This distribution probably occurs through the blood stream, but the resting place of the spirochete is largely in the hematopoietic organs and lymph spaces; for the latter the spirochetes seem to have a special predilection. The subarachnoid space may be considered the great lymphatic sheath of the central nervous system. From it extend the perivascular lymph spaces which accompany the arteries and arterioles as far as the capillaries. The perineuronal spaces are also in direct communication with the subarachnoid space; consequently the spinal fluid, which may be considered the lymph of the central nervous system, is in intimate relation with both the vascular supply and the nerve cells.

The portion of the central nervous system which is most frequently involved in syphilis is the base of the brain between the peduncles and optic chiasm. This is the site of one of the large subarachnoid cisterns, and from this region the process easily extends along the cranial nerves and arteries. Microscopical examination

of the involved vessels shows that the most intense round cell infiltration is in the adventitia, with an endarteritis as a secondary process. In a case of early syphilitic meningoarteritis, Strassmann (1) showed that the spirochetes were most numerous in the adventitia and only occasionally present in the intima. This distribution is probably a result of the mode of invasion of the microorganisms, viz., through the perivascular lymph spaces. This peculiar vessel change, which is seen in all parts of the central nervous system, in all stages of the disease, undoubtedly indicates that the chief mode of extension of the infection through the nervous system is by the perivascular lymphatics, rather than through the blood stream.

Whether tabes is a primary degeneration of the columns, or a degeneration secondary to a localized meningitis, is not decided. The fact remains that in a majority of cases there is evidence in the spinal fluid of an irritative process, and microscopical examination frequently reveals a localized meningitis in the region of the radicular portion of the spinal nerves and corresponding portion of the cranial nerves.

Because of the peculiar anatomical conditions in syphilitic meningoarteritis, the treatment by ordinary means is quite difficult. This is probably due to the fact that there is imperfect application of the therapeutic agent through the blood stream. The most intense process is bathed only by the spinal fluid, into which there is little, if any, excretion of curative agents. Even so highly diffusible a drug as potassium iodide is not normally excreted into the spinal fluid, and the work of Flexner (2) in epidemic cerebrospinal meningitis, of Wollstein (3) in influenzal meningitis, and Lamar (4) in pneumococcus meningitis, has demonstrated that in these diseases it is necessary to introduce the specific therapeutic agent directly into the subarachnoid space to obtain curative results. The same laws, doubtless, are operative in syphilitic meningitis.

There is considerable evidence that the blood serum of treated syphilitics has curative powers. Taege (5) noted that the milk of a syphilitic mother who had received treatment with salvarsan had a marked curative effect on her syphilitic child. By means of seven subcutaneous injections of serum from salvarsan treated patients, Meirowsky and Hartmann (6) were able to clear up the

cutaneous lesions in a congenital syphilitic child. Plaut (7) similarly treated several adults. Among these were two patients with primary lesions, one with an ulcerated throat, and one with an old, deep ulcer of the tongue. In all there was a marked improvement, but in none a complete cure. Controls treated with normal serum were unaffected and those treated with the serum of mercury treated patients were but little improved. Gibbs and Calthrop (8) treated a patient with severe secondary symptoms, using serum from salvarsan treated patients. The serum was obtained from cantharides blisters. This patient had lost eight and one half pounds in eleven days before the treatment was started. With four subcutaneous injections there was a rapid resolution of lesions, and the lost weight was regained in sixteen days. At the same time the Wassermann reaction decreased in intensity. It is difficult to state whether these beneficial effects are due to antibodies or to small amounts of salvarsan which have been changed in the serum to a maximal therapeutic form. In such serums we have an ideal preparation for direct introduction into the spinal canal.

For the past eight months we have been studying the therapeutic effect of such serums when injected intraspinaly into patients with tabes and other syphilitic affections of the central nervous system. At first the serum was obtained by withdrawing blood from the patient on the day following a salvarsan treatment. Gradually the time has been shortened so that now the blood is withdrawn an hour after the intravenous injection of salvarsan or neosalvarsan. Abelin (9) has shown that his diazo reaction for salvarsan is not present more than two and one half hours after treatment. We have used the serum of syphilitic patients to make serum agar culture media for the growth of *Treponema pallidum* after Noguchi's (10) method. Cultures in media made with serum obtained before treatment grew practically as well as in that made with normal serum, while in media made with serum obtained an hour after intravenous injections of salvarsan, the spirochetes developed much more slowly or not at all. In media made with serum obtained in six to twenty-four hours after treatment, the growth approximated that in normal control tubes. From these experiments it seems that the serum has the greatest inhibitory action shortly after the injection of salvarsan.

TECHNIQUE.

Our method is to withdraw blood¹ after intravenous injections of salvarsan or neosalvarsan, separate the serum, and on the following day dilute it to forty per cent. with normal saline. It is then heated at 56° C. for one half hour. By means of lumbar puncture, fifteen c.c. of spinal fluid are withdrawn and then thirty c.c. of the diluted serum, warmed to body temperature, are slowly injected into the sub-arachnoid space. The foot of the bed is raised for about an hour after the treatment. Following the injection, there is frequently a slight rise in temperature, and in tabetics there are often lightning pains in the legs. These pains are more violent in the patients who have previously shown irritative symptoms. In patients other than tabetics, there is very little discomfort beyond occasional headache. The reaction has usually passed off by the following day, when the patients are allowed to be up and about. Several of the patients have spent only two days a week in the hospital; during the rest of the time they have gone about their usual vocations. At first the intervals between treatment were seven days, but it was found that the reactions following injection were too severe with such short intervals. Lately, two or three weeks have been allowed to elapse between the injections.

RESULTS.

The results of some of these treatments are as follows: Four tabetics had received repeated intravenous injections of salvarsan, in addition to mercurial treatment, with resulting symptomatic improvement and reduction in the cell count in the spinal fluid. Upon instituting intraspinal injections of their own serum obtained shortly after intravenous injections of salvarsan, the cell count promptly fell to normal, the globulin decreased in amount much more rapidly than previously, and in two of the patients the Wassermann reaction² in

¹ The blood is drawn directly into large tubes by means of a MacRae venous puncture needle.

² In performing the Wassermann reactions, one half the quantity of the various reagents originally described in the Wassermann reaction are used. In the spinal fluid quantitative estimations of the antibody content are made; 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 c.c. of the fluid are the quantities of spinal fluid employed. It has been found that reactions are frequently positive with the large quantities when they are negative with the smaller amounts.

the spinal fluid became negative, even when 0.5 c.c. of fluid was used. In the other two patients the treatment had little effect on the Wassermann reaction. Another patient with tabes was treated from the beginning by combined intravenous and intraspinous methods. With five treatments in two and a half months, there was a drop in cells in the spinal fluid, from 130 to nine, a disappearance of the Wassermann reaction in the spinal fluid, and considerable decrease in globulin. During this time there was also symptomatic improvement.

It might be contended that in these patients the beneficial effect was due to the intravenous treatment. The fact that improvement in the condition of the spinal fluid after the institution of intraspinous treatment was more marked and more rapid than before, is at least suggestive that the change in the form of treatment had some effect. To study this effect we have used intraspinous treatment alone in certain patients. The first, a well marked tabetic, had eleven injections during a period of five months, during which time the cell count dropped steadily from forty-two to ten in a c.mm., the globulin reaction changed in intensity from a heavy precipitate to a faint haze, and the Wassermann reaction, which at first was strongly positive in 0.1 c.c. of the fluid, became weakly positive in 0.5 c.c. He then received five intravenous injections of 0.3 gramme salvarsan at weekly intervals. At the end of this time the cell count was nineteen, the globulin reaction was stronger, but there was no change in the intensity of the Wassermann reaction. In two patients the effect of injections of normal serum alone has been tried. In one patient with six treatments there occurred a decrease in cells in the spinal fluid from fifty-five to ten and later a rise to seventeen, the globulin reaction became weaker, but the Wassermann reaction became stronger. In the second case during four treatments the cells dropped from fifty-five to fifteen; with four more treatments they returned to fifty. Then serum, which was withdrawn from another patient one hour after treatment, was used and the cells dropped promptly to nine. The effect of this one treatment was more marked than that which followed the eight injections of normal serum. There has been practically no effect on the globulin content or Wassermann reaction. Two other patients, one with

tabes and one with chronic meningitis, having only intraspinal treatments, have shown a steady decrease in cell count in the fluid, but they have been under observation too short a time to determine the final result.

The lymphocytosis and increased globulin content of the spinal fluid are more easily affected than the Wassermann reactions. It has been our experience that it is easier to affect the Wassermann reaction in the spinal fluid in patients in whom the reaction in the blood is negative at the beginning of treatment, than in those giving a positive reaction. In patients with secondary syphilitic meningitis, the intraspinal treatments alone are not sufficient to hold the disease in check. Fortunately, however, in this type of syphilis of the nervous system, the intravenous injections of salvarsan, combined with mercury and iodides, are very efficient, acting more quickly than in the later stages where sclerotic changes in the vessels are more marked.

THE USE OF SALVARSAN AND NEOSALVARSAN.

Naturally the idea of injecting salvarsan directly suggests itself. We have tried this in a number of monkeys. Dilutions of one to 100 to one to 10,000 have been combined with monkey serum and injected intraspinaly. With the lower dilutions lumbar puncture two days after the injections showed marked cellular reactions in the spinal fluid. In one animal permanent paralysis of the legs occurred. With all dilutions the reactions seemed too marked to warrant the intraspinal application of salvarsan in patients. This irritation may be due to the alkaline condition of the drug, for intraspinal injections into monkeys of neosalvarsan, diluted with normal monkey serum, have caused much less marked reactions. Dilutions of one to 5,000, one to 10,000, and one to 30,000 have been injected with only moderate cellular reaction; in fact, only a little more marked than was shown by the control animal in which diluted monkey serum alone was used. The cells in the fluid were largely endothelial, a type of reaction which we have observed in the spinal fluid of a patient after an intraspinal injection of normal human serum.

In one patient we have injected 0.5 milligramme neosalvarsan,

diluted with twelve c.c. of normal human serum and eighteen c.c. of normal saline, intraspinaly, with practically no subsequent reaction. Ten days later he was given one milligramme neosalvarsan, diluted in the same manner, intraspinaly. There was no reaction beyond slight lightning pains, lasting a few hours. Perhaps, by this means, we shall be able to reinforce the good therapeutic effect of serum obtained after intravenous treatment.

CONCLUSIONS.

While the series of cases here reported is small, we feel that the results up to the present are of sufficient value to warrant a continuation of the work and its institution on a larger scale. Doubtless the best results can be obtained from the intravenous treatment with salvarsan or neosalvarsan, combined with intraspinal injections of the patient's own serum, possibly with the addition of small amounts of neosalvarsan. It is hoped that the treatment here outlined will be of aid in these forms of syphilis, the treatment of which has formerly been so unsatisfactory.

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PNEUMOCOCCUS INFECTION AND IMMUNITY.*

By RUFUS COLE, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

During the past few years there has occurred a revival of interest in the study of the nature of infection with the pneumococcus. It will be impossible to review all the discoveries of interest and importance that have been made. The intention is to confine the present discussion mainly to certain observations made by my colleagues and associates and myself, which seem to have some value in enabling us to obtain a clearer conception of the process in pneumonia. Certain of the hypotheses advanced must be considered to be purely tentative and further work may show that different explanations are the correct ones.

Biologically as well as clinically the course of lobar pneumonia consists of these phases: First, there occurs the infection and onset of the symptoms, which events may or may not be simultaneous; second, the clinical disease itself or the intoxication; and third, the recovery or immunization, using the latter word in a broad sense as signifying the onset of a refractory state, whether this be insusceptibility or resistance to the infection or to the intoxication.

THE ONSET OF PNEUMONIA.

In the first place, why does a person contract pneumonia? Is it merely that a pneumococcus, a normal inhabitant of the mouth, possibly riding on a drop of moisture, is accidentally carried into the healthy bronchus, and, after being tossed backward and forward on air currents, finally settles on the mucous membrane of one of the bronchial branches, begins at once to multiply and so induces the

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disease? The disease, according to this view, must be considered purely as an accident due to the pneumococcus reaching the lung. It hardly seems possible that this simple explanation can be the correct one. It is quite probable that pneumococci normally invade the lung in small numbers, at least occasionally. However this may be, the fact is established that in pneumonia the pneumococci are in the lung and multiply there, which is not normally the case. The usual explanation of this occurrence is either that the pneumococci are more virulent than usual, or that the resistance of the host is lowered.

Much work has been done in order to determine whether or not pneumococci obtained from patients with pneumonia are more virulent for animals than those obtained from normal persons. This is an extremely difficult problem to solve, and it is impossible to review all the evidence in regard to this point. An observation made lately, however, seems to have a bearing on the important question as to whether or not pneumococci of low virulence may be responsible for the onset of pneumonia. In making cultures from the blood of pneumonia patients during the past year we have tested the virulence of all the organisms obtained. Most of the organisms so cultivated have been of high virulence for mice and rabbits, but in at least two instances organisms of extremely low virulence have been obtained. While it is possible that in these cases the organisms may have lost their virulence in the body, we know from experiments that exaltation of the virulence of organisms when grown in the animal body is the rule. The experiments of Meltzer and his associates, which I shall mention shortly, also establish the fact that in dogs, at least, pneumonia may be induced by organisms of low virulence.

That a decreased general resistance on the part of the body is present and is responsible for the onset of the pneumonia is possible, but at present the experimental evidence in favor of this view is not convincing.

We have experimental evidence to support the view that the onset of pneumonia occurs because there is a changed condition in the lung which permits pneumococci to grow there. Meltzer, Lamar and Wollstein have elaborated a technic by means of which

it is possible to produce, uniformly and constantly, in dogs, lesions in the lungs which closely resemble, if they are not identical with, lesions found in the lungs of men dying from acute lobar pneumonia. The method consists in the direct injection of quite large amounts of the culture fluid through a catheter inserted into the bronchus. It is a most important fact that these lesions have been produced, not only with organisms having high virulence for rabbits and mice, but also with pneumococci having practically no virulence for these animals. A second important point is that in order to produce these lesions, quite large amounts of culture fluid must be injected, and this must be blown into the finer air-passages, probably, as Meltzer has pointed out, so as to occlude them. This experimental work permits a new conception of the onset of pneumonia. What may first occur in pneumonia is an exudate which may be entirely non-specific in character and may be dependent on circulatory changes due to cold, trauma, etc. The smaller bronchioles being occluded, a true cavity is formed. The lining of the involved air-sacs no longer forms a part of the surface of the body, but lines a true cavity filled with fluid. Pneumococci, being the organisms most commonly present, begin to grow and change the character of the fluid so that it is irritating. An observation by Gillespie shows that the reactions induced by the growth of pneumococci may be quite different, depending on whether free oxygen be present or not. When pneumococci are grown on the surface of serum agar directly in contact with the oxygen of the air, the medium is not clouded, or only to a slight extent. Probably little acid is produced. When, however, they are grown in the depths of the medium so that no air comes in contact, there occurs a marked clouding of the medium.

There is much evidence against the old view that the lesions of lobar pneumonia begin uniformly throughout an entire lobe. Clinical evidence is decidedly against this. Moreover, examination of the lungs of persons dying of lobar pneumonia shows that extension is constantly going on. The extension is not uniform, but is patchy and lobular. The classical description of the lesion of lobar pneumonia in an exudative stage, a stage of red hepatization and one of gray hepatization, is responsible for this old conception. Supported by all these observations, we may conceive that the pneu-

mococcus is not the first cause of pneumonia, but that pneumonia probably arises because in a small portion of the lung a non-specific exudation occurs, and later the pneumococci grow in this and produce irritating substances. From this small focus, or from a few or many small foci, extension occurs until the entire lobe is involved. As the pleura forms a natural barrier to extension, the process tends to become lobar.

THE CLINICAL DISEASE.

The clinical features of pneumonia are those of an acute intoxication. The pulmonary changes are not essential to the condition since pneumococcus septicemia, entirely apart from lung involvement, may exist. Moreover, severe symptoms may arise when the pulmonary lesions are comparatively slight, though this is not usually the case. We have been much impressed with the fact that extension of the local process is associated with a continuation and increase of the symptoms, so that it seems improbable that the general symptoms are entirely independent of the extent of the local lesions. The most severe symptoms, however, arise when, instead of the process being entirely a local one, a general infection supervenes.

There have been several theories to explain the intoxication. It has been suggested that it may be due to the absorption of the products of digestion of the local exudate. But we know that the patient may be free of symptoms when this process is most active, namely, following the crisis. So far, all attempts to discover a toxin in the culture fluids of pneumococci have been unsuccessful. But it is possible that the metabolic products of the growth of pneumococci within the body are different from those formed when the organisms are grown in the test-tube. To test this point, rabbits were profoundly infected with pneumococci. Just before death they were bled, the serum was passed through a Berkefeld filter and the filtrate was then injected into a second series of rabbits. No signs of intoxication could be detected. It is well known that pneumococci, when grown in a medium containing carbohydrate, produce acid, and it is possible also that by the growth of the organism in the body the normal metabolism may be so altered that

the normal reaction of the body tissues may be changed. Complete metabolic studies and studies of the oxygen and carbon dioxide content of the blood, carried on by Peabody, have not tended to support this point of view. A mild grade of acidosis occurs in all febrile conditions, but the evidence seems to indicate that the symptoms in pneumonia represent more than an acidosis.

In our experimentation we have turned to the isolation of toxic substances directly from the bodies of the bacteria. Years ago Pfeiffer showed that the killed bodies of certain bacteria are toxic when injected in considerable amounts. To these so-called endotoxic substances have been loosely ascribed the symptoms in a large number of infectious diseases. Pneumococci, when killed by heat, have very little toxicity. It has been shown by Friedberger, however, that if bacteria are first treated with immune serum, then centrifugalized, and these so-called sensitized bacteria are then treated for a number of hours with serum containing complement at 37° C., a change, supposedly digestive, occurs and the resulting extract is toxic. Owing to the fact that the death resulting from the injection of this extract into guinea-pigs is like that seen in anaphylactic death, Friedberger gave to this substance the name "anaphylatoxin." He thinks that the substances in question are identical with those producing death in anaphylactic shock following the second injection of horse serum. Based on this experiment, Friedberger and others have developed a theory of intoxication in the various infectious diseases, namely, that the bacteria growing in the body stimulate the production of antibodies, and that these sensitize the bacteria, which are then acted on by the complement, and the intoxicating substances result.

We, as well as others, have repeated these experiments with pneumococci and have found them correct, but differences of opinion exist as to their interpretation. It has been shown that under certain circumstances similar results may be obtained by the action of normal serum on the bacteria without previous treatment with immune serum. Dold and also Rosenow have shown that if bacteria are merely allowed to stand in salt solution for from twenty-four to forty-eight hours, so as to undergo what has been termed autolysis, the resulting extract is toxic also. We have also repeated these ex-

periments with pneumococci, injecting the extracts into a large number of guinea-pigs, and have found that under certain circumstances such extracts are toxic, but that their action is inconstant and that it is extremely difficult to determine the exact conditions under which such extracts will be uniformly toxic. Instead of dying acutely, many of the animals die in from three to eight hours, and at autopsy marked hemorrhages are found in the cecum and stomach, and punctate focal hemorrhages are present in the lungs. Based on the experimental production of toxic autolytic extracts, Rosenow has formulated a theory of virulence. He states that virulent pneumococci autolyze readily and set free the toxic substance; non-virulent ones do not. Therefore virulence depends on the readiness with which pneumococci autolyze. We do not feel that the matter is as simple as this. While it is true in general that the more virulent pneumococci do autolyze more readily, yet these two properties, virulence and ability to autolyze, do not run parallel. Typical pneumococci, in our experience, always autolyze, whether they possess high virulence or not. Moreover, if a typical pneumococcus is allowed to grow on artificial culture medium until it has lost its virulence, it still continues to autolyze.

In our efforts to obtain these toxic substances more constantly, in order that more might be learned of their nature, we tried a large number of procedures which need not be mentioned in detail, but finally tried dissolving the bacterial bodies in a dilute solution of bile salts. It is well known that pneumococci are readily soluble in these substances. Much to our surprise, we found that such a solution of pneumococci in bile salts is highly toxic, killing guinea-pigs when injected intravenously within a few minutes. In the experiments the pneumococci were grown in bouillon, washed once in physiologic salt solution, and made into an emulsion in as small an amount of salt solution as possible. The latter procedure is due to the experimental observation that the solubility of pneumococci in bile solutions is dependent on the concentration of the bile in the solution, and not on the relation of the number of pneumococci to the amount of bile present. After solution has occurred, the dilution may then be made to any degree desired. In the experiments a 2 per cent. solution of sodium cholate has been employed, 0.2 c.c.

of which is sufficient to dissolve the bacteria from 100 c.c. of bouillon culture, if the bacterial emulsion be sufficiently concentrated. Many times the amount of sodium cholate used for the single injection may be injected into a normal animal without producing symptoms. It is necessary to use considerable amounts of the dissolved bacterial substance, but not more than needs to be employed in producing an active amount of Friedberger's so-called anaphylatoxin, or in producing a lethal dose of the so-called autolytic extract. Not only is this solution toxic for guinea-pigs, but it also produces acute death in rabbits when injected in proper amounts. The symptoms produced in rabbits are exactly like those described by Auer as occurring in acute anaphylactic shock in rabbits.

It does not seem important to insist on the relation of the toxic substance to anaphylaxis except in connection with the fact that this toxic substance may be produced by allowing the mixture containing bacteria and bile salts to remain at 37° C. for as short a time as ten minutes, or on the ice at 4° C. for thirty minutes. It is generally accepted at the present time that the toxic substance responsible for acute anaphylactic shock is a product arising from the splitting of the protein. If we admit that the intoxication arising from the injection of the bacteria treated in these various ways is identical with that in anaphylactic shock, we must consider that some other explanation of the nature of the intoxicating substance in anaphylaxis must be probable, for it seems hardly possible that a digestive process could be effectual, acting at such temperatures in such short spaces of time. It would seem more likely that the substances concerned are preformed in the bodies of the bacteria and are set free by the solution of the bacterial wall. It would thus seem that in this instance the conception of Pfeiffer of an endotoxin is more likely to be correct than the present conception that such substances are digestive products. In any case, an active poison may now be readily and constantly produced from the bodies of pneumococci. It may or may not be that responsible for the symptoms in pneumonia. This substance is labile, being destroyed at 60° C. for one hour. It not only produces acute death in rabbits and guinea-pigs, but by regulating the dose, death may be induced at different inter-

vals. In the less acute death, hemorrhages into the peritoneum are common, frequently there is an acute nephritis, and the liver is pale.

MODE OF RECOVERY.

The third problem in pneumonia relates to the outcome of the disease. It would seem that in pneumonia, with its sudden crisis—one of the most startling and dramatic events that confront the physician—an ideal opportunity would be offered to learn the nature of the process of recovery; but up to the present little evidence as to the nature of crisis has been accumulated.

It has been thought by some that the crisis is only the general manifestation of the onset of resolution. The theory supposes that in the consolidated lung absorption is constantly taking place, that the exudate is under tension, and that when there has been a sufficient setting free of ferment by the breaking down of leukocytes to induce lysis, tension is relieved, absorption stops and the symptoms disappear. Instead of the surgeon inserting a knife, Nature injects a ferment. This is a fascinating theory, but has little to support it. The crisis often occurs before any signs of resolution may be discovered; indeed, resolution may occur long after all symptoms have disappeared.

In case the toxic substance previously discussed does bear some close relation to that involved in the production of acute anaphylactic shock, it is conceivable that the crisis may be merely the onset of a refractory state analogous to that seen after acute shock. As we are not yet in a position to draw any such conclusion, this is offered merely as a suggestion.

Naturally the main attempt to explain the crisis, as well as recovery by lysis, has consisted in the attempt to demonstrate antibodies in the blood-serum. So far the attempt to demonstrate an increase of the ordinary bactericidal substances which act in conjunction with complement has been successful. Attempts have been made, by combining leukocytes and serum, to show an increase of bactericidal or possibly phagocytic power, but these studies are not free from objection. Most of them have been made with organisms of low virulence. In the experiments in which plating methods were employed, the possibility of agglutination of the cocci

has not been excluded. The experiments of Neufeld are of more importance as showing an increase in immune substances in the blood of patients following recovery from pneumonia. These results were obtained by testing the protective power of the serum for mice against a known lethal dose of pneumococci. He was able to show that while normal human serum had no such protective action, that obtained from patients following the crisis did have such an action. Certain writers, as Strauss and Seligmann and Klopstock, have not confirmed these results.

During the past year Dochez has studied the blood of patients suffering from pneumonia to settle this point and has obtained results which, in general, confirm those of Neufeld. The technic of the experiments was as follows: Specimens of blood were obtained at frequent intervals during the course of the disease, and also at the time of crisis and during recovery. These specimens were all kept on ice until the final specimen was obtained. Then, on the same day, the protective power of all these samples of serum was tested by mixing constant amounts with varying doses of pneumococci and injecting the mixtures into mice. A large number of mice were required, as many as 100 for a single experiment. The organisms used in most cases were those obtained from the patient whose serum was being tested. If they were not virulent when isolated, they were rendered so by passage through animals. The serum from fourteen cases was studied in this way. In one case the serum two days before crisis showed no protective power. Three hours after the crisis 0.2 c.c. of the serum protected a mouse against 0.0001 c.c. of the culture. In this case 0.000001 c.c. of the culture uniformly killed when given alone, or when mixed with the serum obtained before the crisis. The serum obtained two days after the crisis protected a mouse against 0.001 c.c. of the culture. Serum obtained seven days after the crisis showed no protective power.

If this were a constant finding one might conclude that the development of protective immune bodies in the blood of the patient with pneumonia was the cause of crisis, or at least that the crisis was associated with such a change; but this development of immune bodies in the blood serum cannot be demonstrated in all cases. In

fact, among the fourteen cases studied, no other showed such a typical curve as this. In certain cases the appearance of immune bodies did not occur until several days after the crisis had occurred, and in other cases no appearance of immune bodies could be demonstrated. In the majority of cases, however, there occurred an increase in the immune bodies of the blood at or about the time of crisis, and this increase usually persisted for over a week or ten days. The irregular results obtained make it seem hardly possible that the crisis is directly dependent on this one factor alone. From clinical observation it would seem much more likely that the crisis represents a kind of neutralization of the intoxication rather than a destruction of the bacteria existing within the body.

It is nevertheless true that the presence of bacteria in the blood apparently bears some relation to the outcome, for while in the patients that recover the bacteria are absent, or present in small numbers in the circulating blood, in the cases that go on to a fatal termination bacteria are usually present in large numbers. Moreover, while the virulence of the organisms concerned seems to play but a slight rôle in the onset of the disease, this factor is apparently an important one in the outcome. In practically all cases in which large numbers of highly virulent organisms were present in the circulating blood, death resulted. On the other hand, in several cases in which the organisms in the circulation were of low virulence, recovery ensued. It therefore seems probable that while the crisis may result from a neutralization of the intoxication, the possibility of this phenomenon is dependent on the power of the body to overcome the vegetative functions of the bacteria. At the present time, at least, our only definition of virulence as regards pneumococcus must be power to vegetate within the body. In other words, the virulent organism has become adapted to this environment. This adaptation is apparently a property both easily acquired and easily lost by the pneumococcus. It is evident, then, that the crisis in pneumonia is still an obscure phenomenon, and at the present time it cannot be stated positively whether it represents a destruction of the bacteria, a neutralization of the poison, a kind of anaphylactic shock, or a combination of all these phenomena.

ACTION OF IMMUNE SERUM.

Finally, we may briefly consider the newer work concerning specific therapy, especially treatment with immune serum. Observers who have attempted to produce active immunity to pneumococci have usually found little difficulty in doing so. By the injection of living cultures intravenously in horses, as the method was first described by Neufeld, we have succeeded in so immunizing a horse that it will stand as much as 2,500 c.c. of the virulent culture. But while many observers have succeeded in producing an efficient active immunity in various animals, more difficulty has been encountered in producing passive immunity, especially in producing a serum having curative value. When injected together with the culture, the serum which we have employed has been so active that 0.2 c.c. will protect a mouse against 1 c.c. of a culture of which 0.000001 c.c. kills—a protection against 1,000,000 lethal doses; but if the lethal dose of pneumococci is given first and the serum injected only a few hours later, it is difficult to protect, no matter how much serum is injected. This is the stumbling-block in the whole question of treatment with immune serum. Various explanations have been offered. Neufeld, who has done the most valuable work lately in connection with this question, has endeavored to show that the failure to cure is due to the fact that sufficient serum is not employed. He bases this view on certain experiments which he has performed, which show that as one injects mixtures of serum and bacteria into a series of mice, gradually reducing both in the same proportion, a point is reached where the small amount of serum injected fails to be efficacious. He therefore thinks that the reason why our immune serums have failed to cure is that too small amounts, in proportion to the body weight, have been employed.

Dochez has performed experiments which seem to indicate that there may be another explanation for the failure of immune serums to be curative. He has studied the results obtained by injecting mixtures of immune serum and culture into a series of mice, not only decreasing the amounts of serum and bacteria in the same proportion, but also gradually increasing the amount of bacteria injected, and he finds that as one increases the amount of bacteria it is necessary to inject proportionately more and more of the serum

in order to protect, and that finally a point is reached where no amount of serum, however great, is sufficient to save the animal. This seems to indicate that, in addition to the presence of immune bodies contained in the serum administered, it is necessary for the body of the infected animal to play an active part, and that where the infection is very severe the body is unable to react to a sufficient degree.

In pneumonia we are dealing not with a mild infection but with a most severe one, in which the body contains an enormous number of organisms. It seems probable that the reason the serum is not efficacious in these cases is not that a sufficient amount of immune body is not being given, but that the body is not able to complete the action of the immune substance. If we are to make such serums efficacious in treatment, we must find some method of increasing this completing action of the body. Since we apparently do this in active immunization, we may be able to obtain curative effects by combining active immunization, that is, vaccination, with the supplying of immune bodies—the administration of immune serum. It becomes more and more evident that such methods should be carefully worked out on animals before being applied to patients. By careful animal experimentation along this line it is quite possible that favorable results may be obtained even with the resources we now possess.

There is one further point in regard to the use of immune serums which should be borne in mind. Our experiments with blood-cultures have shown that our univalent serum, that is, one produced by the injection of a single strain, has been efficacious in protecting animals against about only 40 per cent. of the cultures obtained from the circulating blood of patients. This means that at least 60 per cent. of cases are due to organisms other than those of the type strain. Whether a polyvalent serum will enable us to overcome this factor is not certain.

In regard to the results of the practical application of specific forms of treatment, but little need be said at the present time, as so far the value of none of them has been sufficiently established. Interesting observations on the specific treatment of experimental pneumococcus infections by means of mixtures of soap, serum and

boracic acid have been made by Lamar. This work is based on sound experimental evidence, but so far has not received practical application. Leukocytic extracts, vaccines and watery extracts of bacteria have been employed as therapeutic measures. Their exact value still remains to be determined. Moreover, Morgenroth has prepared a derivative of quinin, ethyl-hydrocuprein-hydrochlorate, which is said to have curative value in experimental infections and has already received some clinical study. Unfortunately, several cases of amblyopia have resulted from its use.

This report and brief review of some of the work that has been done in connection with pneumonia during the past few years shows that there is considerable interest in the subject and that much work is being done, and while it is difficult at the present time to see exactly where the solution of the whole problem will lie, nevertheless the outlook is not without hope for arriving at a better understanding of the nature of the process, and possibly for obtaining a specific cure.

A STUDY WITH THE ELECTROCARDIOGRAPH OF THE MODE OF DEATH OF THE HUMAN HEART.*

By G. CANBY ROBINSON, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

PLATES 34-38.

The changes in the mechanism of the heart-beat which occur at the time of death have been repeatedly observed experimentally in animals, and from these observations it has been generally believed that that part of the heart in which the property of rhythmicity is the most highly developed is the part that ordinarily continues to beat longest as the heart dies. The evidence seems conclusive that it is in the region where the superior vena cava joins the right auricle that rhythmicity is most highly developed, and that it is in this region that the stimulus of the heart-beat originates. Here it is, in other words, that the cardiac pace-making region is situated, and this function belongs apparently to the specialized tissue found along the sulcus terminalis, which constitutes the node of Keith and Flack.

Koch¹ has questioned the belief that this region in the right auricle, the ultimum moriens, is the last part of the heart to die in man, and he bases his objections on observations on four human fetal hearts which returned to activity after the chest was opened soon after birth. He believed that the region of the coronary sinus was the last part of the human heart to die. Hering² criticizes his findings and says that the incisions made in the hearts were responsible for the lack of agreement between Koch's observations and his experiments.

Rohmer³ made electrocardiographic studies of fatal cases of diphtheria and reported that in three complete dissociation of auricles and ventricles occurred. He found an unusual form of ventricular complex which he thought was due to damage to the myocardium. His paper with curves has not as yet been published. Koch and Rohmer have furnished the only observations which deal directly with the changes in the mechanism of the dying human heart.

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¹ Koch, W., *Beitr. z. path. Anat. u. z. allg. Path.*, 1907, xlii, 203.

² Hering, H. E., *München. med. Wchnschr.*, 1909, lvi, 845.

³ Rohmer, *München. med. Wchnschr.*, 1911, lviii, 2358.

The present study is based upon electrocardiographic records obtained from seven patients before and during the actual stoppage of the heart. There were two cases of poliomyelitis, one of pneumococcus meningitis, and four cases of lobar pneumonia. Death in the cases of poliomyelitis occurred from paralysis of the respiratory muscles, the children being otherwise in relatively good physical condition. As wires run throughout the hospital, records could be taken without moving or disturbing the patients in any way. The second lead was used in each case, the German silver electrodes being attached to the right arm and left leg by bandages wet with salt solution.

I sought to obtain records of the cardiac mechanism just before the occurrence of death and to continue taking records at frequent intervals as long as any cardiac activity persisted. There were naturally many failures to obtain records, especially when fatalities occurred suddenly; while success in some instances followed only after hours of careful watching with the electrodes in place and the galvanometer in operation.

Clinical death was considered to have occurred when respiration finally ceased, when no heart sounds could be heard, and when muscular relaxation and the general appearance of the patient indicated to the physician that death had occurred. Not until all these conditions were fulfilled was the patient considered dead. The electrocardiograms have been analyzed in order to determine the changes in the mechanism of the heart-beat in relation to the time of clinical death in each case. The rate of the heart-beat has been calculated; the conduction time from auricles to ventricles (P-R time), and in some cases through the ventricles as indicated by the duration of the QRS complex, and the length of ventricular systoles (R to end of T) have been measured in seconds. The heights of the various waves have been measured in millimeters and the results of these analyses are seen in the tables.

Case 1.—A child, eighteen months old, suffered with poliomyelitis and died of paralysis of the respiratory muscles. The heart sounds could be heard occurring regularly for three minutes after respirations ceased, and clinical death is considered to have occurred when the heart sounds were no longer audible. The rate of the heart-beat gradually declined from 132, eleven minutes before death (figure 1), to 45, ten minutes later. The cessation of respiration produced no

TABLE I.

Case 1. Age 18 months. Poliomyelitis. Curve No. 134.

Number of curve.	Number of figure.	Time.	Rate per minute.		Conduction time in seconds.		Length of ventricular systole in seconds. Q to end of T.	Height of waves in mm.			
			Auricular.	Ventricular.	P-Q.	Q to end of R.		P.	.	T.	
134.3	1	11 min. a.m.*	132.0	132.0	0.10	0.057	0.24-0.26	1.4	11.5	2.0	
		8 min. a.m.	113.0	113.0	0.11	0.053	0.25	1.3	11.2	2.0-2.5	
		5 min. a.m.	59.0	59.0	0.09-0.10	0.066	0.30-0.31	1.3	10.5	3.2	
		4 min. a.m.	54.7	54.7	0.12	0.066	0.31	1.3	10.0	3.5-4.0	
		3 min. a.m.	54.0	54.0	0.12	0.066-0.077	0.30-0.315	1.0	10.0	3.0	
		Respiration ceased here without affecting electrocardiogram.									
134.11	2	1 min. a.m.	45.0	45.0	0.14	0.068	0.34	1.0	9.0	2.5	
		8½ min. p.m.	—	22.6	—	—	0.26	—	11.0	5.0	
134.12	3	10 min. p.m.	Auricles not beating.		—	0.083	0.29	1.0	9.5-	—	
		Complete block.	39.0	17.3	—	—	—	—	10.0	5.5	
134.14	4	11 min. p.m.	0	15.8	—	0.093	0.30	—	9.0	5.5-6.0	
		Auricles not beating.		—	—	—	—	—	—	—	
134.22	5	11½ min. p.m.	28.5	15.4	—	—	0.29	—	8.0-	5.5	
		To show change in complex.						0.27-0.28	—	8.5	5.0-5.5
		11¼ min. p.m.	Occa-sional beat.	16.2	—	—	—	—	8.0	—	
		12½ min. p.m.	Occa-sional beat.	18.2	—	—	0.311	—	6.5	—	
		13½ min. p.m.	Occa-sional beat.	22.2	—	—	0.29	—	6.5	—	
		14 min. p.m.	Occa-sional beat.	26.0	—	—	0.29	—	7.5	—	
		14½ min. p.m.	Occa-sional beat.	—	—	—	0.28	—	8.0	—	
		16½ min. p.m.	Occa-sional beat.	Long pause.		—	0.28	—	8.0	—	
134.22	5	17½ min. p.m.	35.0	—	—	0.28	—	8.0	—		
		No movement of string after 18 minutes p. m.									

*In the tables a. m. stands for ante mortem, p. m. for post mortem.

appreciable effect. The conduction (P-R) time gradually lengthened from 0.10 to 0.14 of a second during this time. Records could not be obtained until eight and a half minutes after death on account of an accident to the instrument. Then the P-wave was absent, indicating cessation of auricular activity, the ventricles beating 22.6 times per minute (figure 2). Ten minutes post mortem the auricles were again beating regularly at a rate of 39 per minute, while the R- and T-waves indicated that the ventricles beat 17.3 times a minute, complete dissociation being present (figure 3). The auricles again ceased beating for a short time and then began again, at first at a rate of 28.5 (figure 4). Afterwards they beat only occasionally and irregularly until sixteen and a half minutes post mortem, when they finally ceased. The ventricles continued to beat until eighteen minutes post mortem and gradually increased in rate from 15.4 per minute, at eleven and a half minutes post mortem (figure 3), to 35, at sixteen and a half minutes post mortem (figure 5). This increase in rate seems to depend, however, at least in part, on the rest afforded the heart by the long pauses that occurred. In this case then the ventricles beat after the auricles had ceased. The curves reproduced from those obtained in this case show the characteristic changes in the ventricular complex which occurred in every case. These changes consist in a decrease in the size of the R-wave, an increase in the size of the T-wave, and a gradual fusing of these two waves. There is no striking change in the duration of ventricular systole in this case. The time of conduction of the impulse throughout the ventricles, as measured from the beginning of Q to the end of R, gradually lengthened from 0.057 to 0.093 of a second. On account of the fusion of R and T, it could not be measured later than eleven minutes post mortem (table I).

Case 2.—A child of four years, with poliomyelitis, died of respiratory paralysis. The time when the heart sounds ceased to be heard was not recorded, but on account of definite changes that took place suddenly in the electrocardiograms corresponding to changes that occurred in other cases at the time of clinical death, the time of these changes is inferred to be the time of clinical death.

For several hours before death the patient breathed with great difficulty, one half of the diaphragm alone supporting respiration. Four hours and a half before death, marked changes in rate occurred in a fairly rhythmic manner (figure 6). Diminution in the size of the P- and T-waves occurred with the periods of slow rate, producing an electrical complex resembling that seen when the vagus tone is raised (Rothberger and Winterberg^{*}). It seems probable that these changes in rate and in the form of the complex are dependent on changes in vagus tone accompanying respiratory movements.

At the time when clinical death is inferred to have occurred, there was marked slowing of the cardiac rate and the auricular activity apparently ceased, the ventricles contracting alone at a rate of 61 per minute. The auricles were seen (figure 7) to become active once more, and when they set the pace of the ventricles again, the rate increased to 90 per minute. The auricles stopped beat-

^{*} Rothberger, J., and Winterberg, H., *Arch. f. d. ges. Physiol.*, 1910, cxxxv, 506.

TABLE II.
Case 2. Age 4 years. *Poliomyelitis. Curves 118-122.*

Number of curve.	Number of figure.	Time.	Rate per minute.		Conduction time in seconds.		Length of ventricular systole in millimeters, R to end of T.	Height of waves in mm.		
			Auricular.	Ventricular.	P-R.	R-S.		P.	R.	T.
119.7	6	60 hrs. a.m.	125	125	0.14	0.067	0.29	2.3	9.5	3.0
		10 hrs. a.m.	91	91	0.13	0.065	0.30	1.3	12.5	3.0
		4 hrs. and 35 min. a.m.	100	100	0.14	0.061-0.068	0.28-0.29	1.0-1.5	9.0	2.0-3.5
121.4	7	There is a marked change of rate at this time. Changes in conduction through the bundle and through the ventricles do not go parallel.								
		Death probably here.	—	61.8	—	—	11.0 mm.	—	5.0	3.0
		90.0	90.0	90.0	—	—	13.0 mm.	—	6.0	2.5
		Rate more rapid when auricles set the pace.	Not active.	58.5	—	—	13.0 mm.	—	5.5	3.0
		$\frac{1}{4}$ min. p.m. $\frac{1}{4}$ min. p.m.	57.6	57.6	—	—	11.0-12.0 mm.	—	—	—
122.2	8	Not delayed.								
		The auricles return and take up the pace in this curve after having ceased beating for 15 seconds. The rate is not altered.								
		5 min. p.m.	56.8	56.8	Delay in P-R time.	—	10.0 mm.	1.5	4.5	3.5
		12 min. p.m.	49.4	About 33 (irregular).	—	—	10.0 mm.	—	—	—
		There is partial block, 3 to 2 rhythm being present. Conduction much delayed when a beat gets through.								
		17 min. p.m.	—	21.4	—	—	10.6 mm.	—	7.5	2.0
		No evidence of auricular activity; ventricles regular.								
		18 min. p.m.	54.8	18.0	—	—	10.0 mm.	—	7.	3.0
		19 min. p.m.	39.2	14.7	—	—	9.2 mm.	—	5.0-6.0	3.0-4.0
		Complete block typical.								
		19 $\frac{1}{4}$ min. p.m.	40.0	13.6 (irregular)	—	—	9.7 mm.	—	5.0	3.4
		20 min. p.m.	40.0	Ventricular fibrillation.	—	—	—	—	—	—
		20 $\frac{1}{4}$ min. p.m.	40.0	One last ventricular wave.	—	—	6.5 mm.	—	5.0	—
		20 $\frac{1}{2}$ min. p.m.	38.4	Not active.	—	—	—	—	—	—

* These measurements were made in millimeters, instead of seconds, as the time-marker was not operating.

ing again for about fifteen seconds, and then the ventricular rate again sank to 58.5 per minute. The auricles began again three quarters of a minute after the time clinical death is inferred to have occurred and took up the pace at about the same rate as that at which the ventricles were already beating. The auricles then continued to beat almost constantly, decreasing slightly in rate, and were still active at a rate of 34 beats a minute in the last curve taken, twenty minutes after the inferred time of clinical death. The ventricles followed the pace of the auricles until twelve minutes after inferred clinical death, when, following a period of delayed conduction, partial block (3 to 2 rhythm) occurred. Complete heart-block occurred six minutes later, the ventricular rate being 18 per minute. This rate gradually decreased and at nineteen minutes after inferred clinical death, the ventricles beat irregularly 13.6 times a minute. One minute later ventricular fibrillation set in (figure 8), after which one ill formed ventricular complex occurred. There was a gradual but not marked shortening of the ventricular systole, as was indicated by the length of the ventricular complex in the electrocardiograms, and the characteristic change in its form occurred (table II).

Case 3.—A child of nine months, who died of pneumococcus meningitis. No tracings were taken until four minutes after clinical death, at which time the heart was beating at a rate of 40 per minute. The electrical complex was at this time abnormal, there being a diphasic P-wave representing the auricular activity, while the T-wave was much exaggerated and almost fused with the R-wave. The conduction (P-R) time gradually lengthened in the record made at this time, from 0.22 to 0.28 of a second. At five minutes post mortem the rate was 28. The P-wave had become negative and very small, while the conduction time had shortened, varying from 0.145 to 0.16. This seems to indicate that the point of origin of stimulus formation had moved to a point in the auricles nearer the atrioventricular junction. The R- and T-waves approached each other. At the end of the record a long pause of over six seconds occurred. At six minutes post mortem two beats occurred at the same rate as before. Both auricular and ventricular complexes were seen. In the latter the R- and T-waves were partly fused. No evidence of cardiac activity was seen in records made during the next few minutes, so in this case cardiac activity ceased six minutes after clinical death, the auricles and ventricles stopping synchronously (table III).

TABLE III.

Case 3. Age 9 months. Pneumococcus Meningitis. Curve 138.

Time.	Rate per minute.	Conduction time in seconds. P-R.	Length of systole in seconds. R-T.	Height of waves in mm.		
				P.	R.	T.
4 min. p.m.	40	0.22-0.28	0.24-0.28	1.3	6.0	5.0
	Diphasic P-wave.					
5 min. p.m.	28	0.145-0.20	0.20-0.27	—	6.0-7.5	3.5-4.0
	P-wave is negative and nearer the ventricular complex than before.					
6 min. p.m.	27	0.16	0.25	—	4.5	—
	Only two beats.					

Case 4.—A man of thirty-seven, who died of lobar pneumonia. The heart was beating at a rate of 136 per minute sixteen and a half hours before death and the electrocardiogram shows that the rhythm was regular except for an occasional auricular extrasystole. The first post-mortem record was not made until four minutes after clinical death, when the heart was beating irregularly and the electrocardiogram showed a very unusual form of arrhythmia (figure 9). In the second group a positive P-wave was followed by a small R-wave, the P-R time being 0.15 of a second. Then a negative P-wave occurred, followed by a small R-wave, the P-R time being 0.24 of a second. Then after a long diastole, a large R-wave, which was not preceded by any evidence of auricular activity, occurred. This group was repeated several times. One large R-wave alternated with two small R-waves, the small R-waves alone being preceded by P-waves. Later these were always negative. From six to fourteen minutes post mortem the cardiac rate varied from 43 to 64 a minute in an irregular manner. At first there was no evidence of auricular activity, but at eight minutes post mortem there was a small negative P-wave preceding the R-wave by 0.123 of a second. The P-wave gradually increased in prominence, but remained negative, while the conduction (P-R) time gradually lengthened until it was 0.31 of a second (figure 10). After fourteen minutes post mortem there was no evidence of auricular activity, while the ventricles continued to beat for thirty-five minutes after clinical death. Their rate became gradually slower and

TABLE IV.

Case 4. Age 37 years. Pneumonia. Curve 179.

Number of curve.	Number of figure.	Time.	Rate per minute.	Conduction time in seconds.		Length of ventricular systole in seconds. R to end of T.	Height of waves in mm.		
				P-R.	R-S.		P.	R.	T.
179.2	9	16 hrs. and 37 min. a.m.	136.4	0.13-0.14	0.075	0.315	2.2	9.5	2.0
		4 min. p.m.	78.5	0.15-0.24	0.065-0.08	—	—	9.0-12.5	—
		6 min. p.m.	64.0	A very unusual type of arrhythmia seen. Auricles not active.		0.285	—	7.5	4.0
		8 min. p.m.	43.0	0.123	—	0.250	—	5.5	4.0
		9 min. p.m.	46.0	0.24	—	0.230	Negative P-wave.	6.5	3.5
179.7	10	10 min. p.m.	60.0	0.26	—	0.260	—	6.0	3.5
		14 min. p.m.	54.0	0.31	—	0.240	—	6.0	3.0
		15 min. p.m.	40.6	Last of auricular activity.		0.220	Very long conduction time.		
		21 min. p.m.	36.0	—	—	0.240	—	7.5	2.5
		23 min. p.m.	30.0	—	—	0.240	—	8.0	—
		28½ min. p.m.	26.0	—	—	0.240	—	7.0	—
		Ventricles beating irregularly.				0.240	—	8.0	—
179.23	11	31½ min. p.m.	—	—	—	0.240	—	8.0	—
		33 min. p.m.	13.8	—	—	0.240	—	7.5	—
		33½ min. p.m.	6.3 seconds between the two beats.			—	—	—	—
		35 min. p.m.	1 beat only.			0.350	—	6.0	—

their activity was irregular after twenty-eight and a half minutes post mortem. Only a single but well defined ventricular complex was seen thirty-five minutes post mortem (figure 11). The characteristic fusion of the R- and T-waves took place gradually, while except for the final ventricular complex, which was abnormally prolonged, no marked change took place in the length of the ventricular systoles. In this case was seen the longest post-mortem cardiac activity that was observed (table IV).

Case 5.—A woman of twenty-five years, who died of pneumonia. No records were made until twelve minutes after clinical death, when the heart was beating 40 times a minute. The electrical complex was at this time abnormal, consisting of a fairly well defined P-wave, a deep Q-wave, and a small R-wave already fused with the T-wave. At thirteen minutes post mortem the rate was 35.6 and the conduction (P-Q) time was delayed (0.225 of a second, figure 12). The rate slowed gradually and the conduction time became slightly more delayed until at sixteen minutes post mortem the rate was 24.4 per minute, while the conduction time was 0.24 of a second. At seventeen minutes a long pause occurred, 13.6 seconds of which were recorded. This pause was followed by a very unusual ventricular complex which followed a notched P-wave after a conduction time of 0.28 of a second. The complex consists of a typical left ventricular, or apical complex followed by a right ventricular, or basal complex (figure 13). That these forms of complexes result from left and right ventricular contractions has been demonstrated by Nicolai[†] and others. This unusual form of complex suggests that first the left and then the right ventricle contracted separately. It was followed by a pause, eight seconds of which were recorded. The cardiac activity was again resumed and the heart continued

TABLE V.

Case 5. Age 25 years. Pneumonia. Curve 149.

Number of curve.	Number of figure.	Time.	Rate per minute.	Conduction time in seconds. P-Q.	Length of ventricular systole in seconds.	Height of waves [‡] in mm. T.
149.2	12	12 min. p.m.	40.0	—	—	—
		13 min. p.m.	35.6	0.225	0.31	5.5
		14 min. p.m.	34.0	0.25	0.31	6.0
		15 min. p.m.	30.4	0.24	0.31	6.0
		16 min. p.m.	24.4	0.24	0.33	6.0
149.6	13	17 min. p.m.	Unusual type of complex suggesting hemisystole.			6.5
					0.87	
		18 min. p.m.	30.8	0.24	0.34	5.2
		19 min. p.m.	30.0	0.24	0.33	5.7
		20 min. p.m.	31.0	0.26	0.36	5.0
		21 min. p.m.	24.0	0.28	0.34	5.5
		22 min. p.m.	32.7	0.35	0.36	5.0
		A pause of 22 seconds preceded the foregoing curve.				
149.15	14	23 min. p.m.	20.0	0.375	0.35	6.0
		25½ min. p.m.	After a stoppage of 2½ minutes.			
			33.0	0.42-0.54	0.40	0.35

[†] Nicolai, G. F., *Med. Klin.*, 1912, viii, 322.

[‡] P and R not measurable.

to beat slowly but with marked changes of rate and long periods of complete cardiac standstill. The heart finally ceased beating twenty-five and a half minutes after clinical death. Just before the final record was obtained, a pause for two and a half minutes occurred. After this pause both auricles and ventricles again became active at a rate of 33 beats a minute. The conduction time in this record (figure 14) gradually lengthened until the ventricles failed, the auricles continuing for a short time at the same rate. The conduction time, which was already distinctly delayed at nineteen minutes post mortem, further lengthened and at twenty-three minutes post mortem it was 0.375 of a second. In the final record it increased beat by beat from 0.42 to 0.54 of a second. The form of the ventricular complex changed very little in this case, except that the duration of ventricular systole gradually lengthened (table V).

Case 6.—A woman of thirty-seven years, with pneumonia. Before death, electrocardiograms showed a heart rate of 156, a conduction time of 0.12, and ventricular systole of 0.25 of a second duration (figure 15). The first record obtained one minute after clinical death showed that the heart was beating regularly at a rate of 72 per minute with practically no change in conduction time (figure 16). At two minutes post mortem there was complete dissociation of auricles and ventricles, and groups of ventricular contractions separated by long

TABLE VI.

Case 6. Age 37 years. Pneumonia. Curve 195.

Number of curve.	Number of figure.	Time.	Rate per minute.		Conduction time in seconds.		Length of ventricular systole in seconds. R to end of T.	Height of waves in mm.		
			Auricular.	Ventricular.	P-R.	R-S.		P.	R.	T.
195.2	15	52 min. a.m.	156.0	156.0	0.120	0.09	0.25	1.5-2.5	11.5-12.3	2-3
	14	min. a.m.	155.6	155.6	—	—	—	—	—	—
195.5	16	1 min. p.m.	72.0	72.0	0.128	0.08	0.265	1.5	11.0	3.0
	1	½ min. p.m.	65.4	65.4	0.115	0.08	0.30	1.0	11.0	3.5
195.7	17	2 min. p.m.	Irregular. Occasional auricular activity. Last seen in this curve. Ventricles beating irregularly. Long pauses seen.							
195.8	18	2½ min. p.m.	—	168.0	—	0.08-0.094	0.30	1.5	9.5-10.3	2.5
			Ventricular tachycardia.							
		3½ min. p.m.	—	138.0	—	—	—	—	8.0- 8.5	2-3
			in small group. Ventricles beating in groups and isolated beats without auricles. Long pauses of several seconds occurred.							
195.12	19	4 min. p.m.	—	47.4	—	0.10	—	—	7.0	2.0
		4½ min. p.m.	—	Ventricular rhythm established. Slight arrhythmia.						
195.14	20		Ventricular fibrillation.							
			31.5	—	—	—	—	—	—	—
		5 min. p.m.	Ventricles at first irregular and then rhythmic. Marked change in complex.							5.5
		5½ min. p.m.	—	31.9	—	—	—	—	0.23	6.0
		6 min. p.m.	No beats.							
		6½ min. p.m.	—	27.0	—	—	—	—	0.23	6.0
		7 min. p.m.	One beat.							
			—	24.0	—	—	—	—	—	
		7½ min. p.m.	Two beats only.							5.3

pauses were seen. Auricular activity ceased altogether at this time (figure 17). Two and a half minutes post mortem a group of ventricular complexes occurred at a rate of 168 per minute, showing apparently true ventricular tachycardia (figure 18), while four minutes post mortem the ventricular rhythm established itself at a rate of 47.4 with but slight arrhythmia. Four and a half minutes post mortem ventricular fibrillation of short duration set in (figure 19), and was followed for about one minute by a period of distinct arrhythmia, but at a fairly slow rate. Five and a half minutes post mortem the ventricular rhythm again became regularly established at a rate of 32 per minute (figure 20). The ventricular complexes were markedly altered and showed the characteristic form seen in nearly all the cases observed just before cessation of ventricular activity. The rate then became slower and long periods without cardiac activity occurred. Two last beats, occurring at a rate of 24 per minute, were seen seven and a half minutes after clinical death. This case showed at first a lengthening and then finally a distinct shortening of the length of ventricular systole, which reached 0.33 of a second at five minutes post mortem and then shortened to 0.2 of a second in the last record (table VI).

Case 7.—A woman of thirty-four years, who died also of pneumonia. The electrocardiograms from this case indicated that the auricles had ceased to beat one minute before clinical death, while the ventricles continued for seventeen minutes after the patient ceased to breathe. The ventricles showed marked irregularities in rate, but two minutes post mortem they were beating regularly at 45.8 per minute. They beat at a rate of 107 times a minute thirteen minutes post mortem, showing a bigeminal type of arrhythmia. They beat regularly at a rate of 47, sixteen minutes post mortem, but no evidence of cardiac activity was seen seventeen minutes post mortem. Because the records were not satisfactory, a more detailed analysis is not possible (table VII).

TABLE VII.

Case 7. Age 34 years. Pneumonia. Curve 228.

Time.	Rate.		
1 hr., 6 min. a.m.	120.0	Auricles active.	Ventricles regular.
1 min. a.m.	57.1	Auricles not active.	Ventricles regular.
$\frac{1}{2}$ min. a.m.	51.7		Ventricles regular.
2 min. p.m.	45.8		Ventricles regular.
4 min. p.m.	60.0		Ventricles nearly regular.
The type of ventricular complex changed here.			
11 min. p.m.	60.0	Ventricles beating irregularly.	
13 min. p.m.	107.0	Ventricles beating bigeminally.	
14 min. p.m.	71.0	Ventricles beating irregularly.	
15 min. p.m.	53.6	Ventricles beating regularly.	
16 min. p.m.	47.0	Ventricles beating regularly.	
No movement of string after 17 minutes post mortem.			

SUMMARY.

In four of the seven cases the ventricles remained active from one and a half to eighteen minutes after the electrocardiograms

failed to show evidence of auricular activity. In two cases the auricles outlasted the ventricles and in one case only did the auricles and ventricles stop apparently at the same time. Complete dissociation occurred three times. Some delay in the conduction time was seen in five of the seven cases. In two cases the auricles ceased to beat before evidence of impaired conduction appeared. There was always marked slowing; the slowest independent ventricular rates varied from 13.6 to 47.0. The slowest rates at which the auricles beat regularly varied from 20 to 65 per minute. There was never evidence of auricular fibrillation, although in two cases the electrocardiograms give fairly conclusive evidence that ventricular fibrillation occurred. The ventricles reestablished a regular rhythm after a short period of ventricular fibrillation in one case, while in the other but one ventricular contraction occurred after the appearance of fibrillation.

Characteristic changes in the ventricular complex of the electrocardiograms occurred in all the records. They consisted of a gradual fusion of the R- and T-waves, forming, when the fusion was complete, a large rounded or peaked wave. In some cases the identity of the two waves was not entirely lost. In spite of the marked change in shape of the ventricular complexes, there was often but little change in their duration. In some cases the ventricular systole was shortened at the end, while in others it was prolonged. The change in the form of the ventricular electrical complex indicates that the course of the stimulus and the manner of the contraction of the muscle were abnormal. The fact that the R-wave became gradually prolonged suggests that the conduction of the stimulus through the ventricular walls became delayed as the heart died. The fact that after death there is a continuation of cardiac muscular activity sufficient to cause a difference in electrical potential between the two sides of the body does not necessarily mean that a ventricular systole in the sense of muscular shortening takes place. It has been observed experimentally that well defined electrical complexes may be caused by cardiac activity which cannot be seen or recorded graphically. As the duration of the ventricular complexes characteristic of the dying heart usually does not differ markedly from the duration of the complexes before clinical death, it seems probable



FIG. 5.

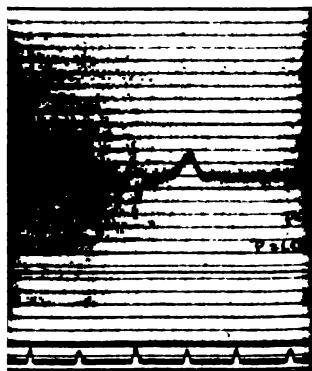


FIG. 6.

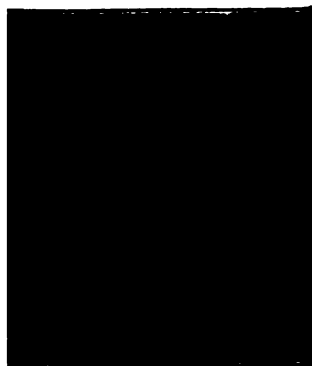


FIG. 7.

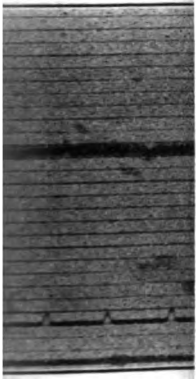
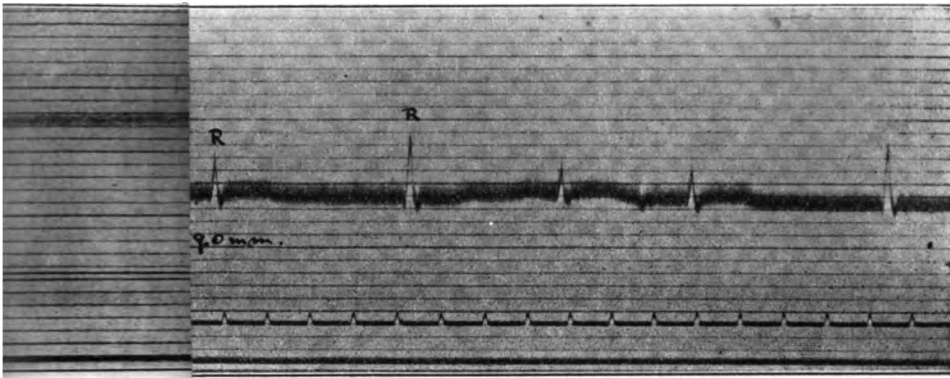


FIG.

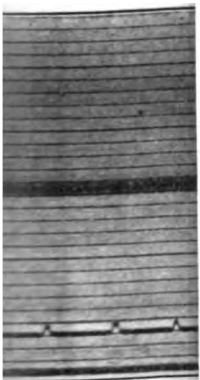
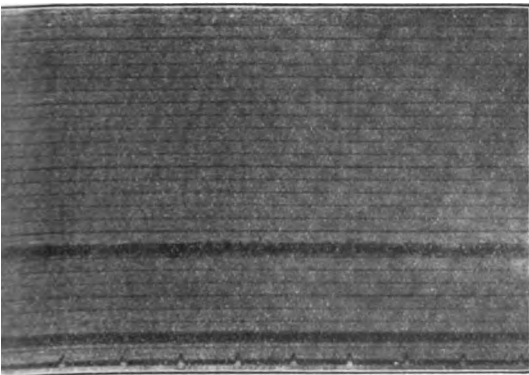
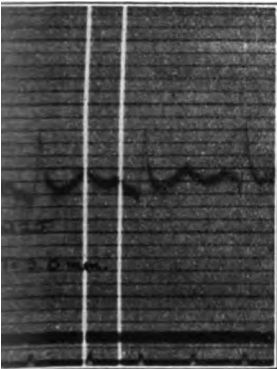
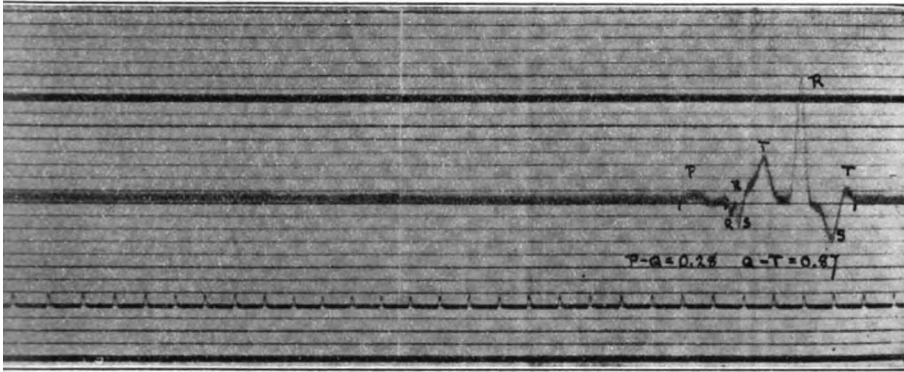


FIG. II.

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PLATE 37.



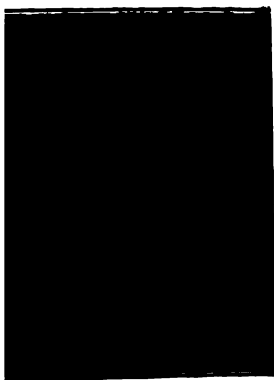
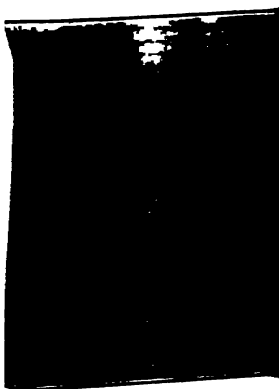
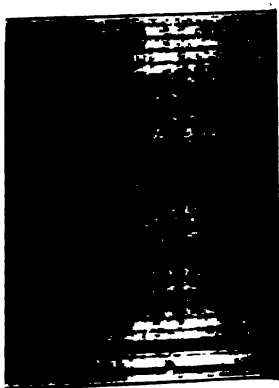


FIG. 18



that the entire musculature of the ventricles participates in the contraction; as definite shortening, or at least a marked change in duration, would be expected if only a part of the ventricular musculature participated in the activity which produced the complex.

CONCLUSIONS.

In acute infectious diseases cardiac activity sufficient to give a definite record with the electrocardiograph may continue in the human heart for some minutes after clinical death has occurred. In the seven cases described the cardiac activity continued from six to thirty-five minutes after all the usual clinical signs of death had occurred. In four cases the ventricular outlasted the auricular activity; in two cases this was reversed; and in one case the two parts of the heart seemed to cease synchronously. Marked slowing of the rate of cardiac activity always occurred and there was usually distinct delay in the conduction time between auricles and ventricles. Complete dissociation was seen in three cases. Ventricular fibrillation occurred in two cases, in one of which the ventricles again established a regular rhythm. Evidence of auricular fibrillation was never seen. Characteristic changes in the ventricular electrical complex occurred in all cases. They consisted of a decrease in the size of the R-wave and an increase in the size of the T-wave, and a tendency to a fusion of these waves. There was usually but little change in the duration of the ventricular complexes as the cardiac activity gradually ceased. The foregoing observations indicate that when death occurs from an acute infectious disease there is no one point in the human heart which may be considered as the "ultimum moriens."

ANAPHYLAXIS TO SALVARSAN.*

By HOMER F. SWIFT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

The use of salvarsan has proved of interest, not only from the standpoint of its curative powers, but also on account of its toxic effects. It is the object of this communication to explain the nature of one of these by-effects.

Practically all drugs with a specific action are poisons. As pointed out by Ehrlich, the relation of curative power to toxicity determines the value of any preparation, and it is the object of the synthetic chemist to furnish a compound with high therapeutic properties as compared to toxic action. In salvarsan we have an active therapeutic agent, but certain toxic properties of the drug have not escaped notice. In fact, the study of the immediate toxic symptoms following intravenous injections has led to an improvement in technic which has rendered possible a much more thorough application of the drug. Wechselmann's discovery, that stale water or saline containing molds or saprophytic bacteria is responsible for the symptoms resembling those of acute arsenical poisoning which formerly were so common following injection of the drug, is one of the most important contributions since the discovery of salvarsan. The use of absolutely freshly distilled and sterilized water has entirely changed our ideas of the toxicity of the drug, so that now it is considered to be even less toxic than mercury.

Several cases of encephalitis hemorrhagica following intravenous injections of salvarsan have been reported. Fischer,¹ in discus-

* Read in the Symposium on Mercury and Salvarsan in the Treatment of Syphilis in the Sections on Pathology and Physiology and Dermatology of the American Medical Association, at the Sixty-Third Annual Session, held at Atlantic City, June, 1912.

1. Fischer, B.: München. med. Wchnschr., 1911, lviii, 1803.

sing a case which he studied, suggested that this lesion might be due to a hypersensitive condition of the patient, since the symptoms followed a second injection. Hoffmann and Jaffé,² and Levens³ have reported cases showing severe intoxication, starting with anaphylactic-like symptoms immediately after a second intravenous injection, and think that these symptoms occurred in association with a state of hypersensitiveness induced by the first injection. Marschalko,⁴ however, in reporting a similar reaction, which followed a first injection of 0.28 gm., considered that the reaction was due to the use of mold-containing water. An experience of ours supports this view. A patient shortly after his fourth intravenous injection of 0.4 gm. went into a state of collapse, which was followed by a high fever, marked gastro-intestinal symptoms, herpes and anuria. The little urine passed the next day boiled solid and contained many casts. The signs of nephritis gradually disappeared, as did the other toxic symptoms, and the patient's condition returned to normal. This case might have been explained as one associated with hypersensitiveness induced by previous treatment, had not the subsequent administration of salvarsan disproved this view. Subsequently the patient had eleven injections of salvarsan. During a period of eight months he had five injections of 0.2 gm. each, followed later by six injections of 0.3 gm. each. Absolutely no toxic symptoms or evidence of renal irritation followed any of these later treatments. The toxic symptoms occurred before we were accustomed to use freshly distilled water, while in the eleven injections following, in which there were no symptoms of intoxication, a more careful technic was followed. It is of interest to note that the above-mentioned cases of intoxication occurred before Wechselmann's discovery.

In florid syphilis one frequently sees a fever and general malaise following the first injection of salvarsan. This picture is probably due to the setting free of some toxic substances from the spirochete.

In addition to these by-effects, a symptom-complex resembling

2. Hoffmann, E., and Jaffé, J.: *Deutsch. med. Wchnschr.*, 1911, xxxvii, 1337.

3. Levens: *Med. Klin.*, 1911, vii, 852.

4. Marschalko: *Deutsch. med. Wchnschr.*, 1911, xxxvii, 1702.

acute anaphylaxis occasionally occurs after repeated intravenous injections of salvarsan. In our experience it has occurred only after repeated injections. Such an attack comes on usually after 30 to 50 c.c. of solution have been introduced. There is a facial expression of anxiety, the patient complains of a feeling of pressure in the epigastrium or cardiac region; this is followed shortly by a sense of suffocation, and a number of times the patients have described a sensation "as though the heart were in the throat." Shortly after this there is a bright red suffusion of the skin of the face, most marked about the eyes; at times, it involves the entire body. In marked cases the eyelids and lips appear swollen. Occasionally the red color changes to a purplish cyanosis. As a rule there is but little change in the pulse-rate, although once it was much increased. The respirations increase slightly in rapidity. The symptoms seem to be largely vasomotor in nature and usually pass off rapidly on stopping the injection.

The stage of the disease does not seem to be an important factor in determining whether the reaction will occur or not. Five of our patients were in the secondary period, two in the tertiary and one had tabes. Three times the first reaction occurred during the fourth injection of a series, twice during the fifth injection, three times during the sixth and once with the seventh. It has recurred with the succeeding treatment in three cases; twice the next treatment did not induce a reaction; once the reaction reappeared at the time of the second succeeding injection following the first reaction. In the patient who showed the most severe reactions, occurring with the fifth and sixth treatment, neosalvarsan was injected two weeks after the last reaction and no symptoms followed. Another patient with a severe reaction during his sixth injection had no reaction with an injection of neosalvarsan a week later. Three times the second reaction was much more intense than the first. Two of the patients showed a skin hypersensitiveness. One, a patient with tabes, had a purpuric eruption on the legs following the first treatment. He also has shown a similar eruption after iodids. The other, a patient with leukoplakia, developed a severe vesicular dermatitis after his eighth treatment. He then was without treatment for eight months, at the end of which time a single intravenous

injection of 0.3 gm. was accompanied by a sensation of pressure in the chest, followed in four hours by a generalized erythematous eruption which later became vesicular. This case is an excellent example of hypersensitiveness induced by previous treatment. In three other patients the hypersensitive condition passed off in five weeks, three, and four months, respectively. In one of these it returned at the time of a fourth injection.

Iwashenzow⁵ has reported a series of fifteen patients who showed similar symptoms. Fourteen of them had the reaction at the time of the fourth injection, and one with the fifth treatment. His treatment consisted of an intravenous injection of 0.2 or 0.3 gm. salvarsan repeated every two weeks. All of his patients had some syphilitic involvement of the central nervous system. He showed by injecting saline alone that the reaction was not due to proteid substances in the saline used for dissolving the salvarsan. Iwashenzow's cases have one feature in common with ours, *i. e.*, the symptoms followed the repeated intravenous injection of 0.2 or 0.3 gm. The interval between treatments in his cases was two weeks, in ours usually seven days. In both series the reaction occurred with or after the fourth injection.

The reactions seem to be anaphylactic in nature because they appear only after repeated injections; the respiratory symptoms and feeling of oppression and the vasomotor manifestations are similar to those observed in anaphylaxis to a foreign proteid. They differ from serum anaphylaxis in that the period of anti-anaphylaxis is shorter or does not exist at all, although this point has not been sufficiently investigated. In two of our patients with very marked symptoms the injection was renewed after ten minutes without any return of the reaction. This might be interpreted as evidence that a condition of anti-anaphylaxis existed.

In recent years there has been a growing tendency to consider hypersensitiveness to certain drugs as an anaphylactic phenomenon. By passively sensitizing guinea-pigs with the serum of patients who showed an idiosyncrasy to iodoform and antipyrin, Bruck⁶ demonstrated the anaphylactic nature of the hypersensitiveness to these

5. Iwashenzow, G.: *München. med. Wchnschr.*, 1912, lix, 806.

6. Bruck, C.: *Berl. klin. Wchnschr.*, 1910, lxvii, 517, 1929.

drugs. Klausner⁷ confirmed these observations with the serum of patients unusually sensitive to both iodoform and iodids. Friedberger and Ito⁸ were able to sensitize guinea-pigs actively with a mixture of guinea-pig serum and tincture of iodine. After an interval of two to three weeks these animals were hypersensitive to the same mixture or to sodium iodid or Lugol's solution. A first injection of Lugol's solution induced a condition of hypersensitivity to iodized guinea-pig serum, but not to sodium iodid or Lugol's solution. The iodine seemed to alter the native serum so that it acted like a foreign proteid. Last year Auer⁹ tried to sensitize guinea-pigs with salvarsan and after a suitable period attempted to induce shock by a second injection of salvarsan. His results were entirely negative.

In approaching this problem, I have mixed salvarsan with guinea-pig serum and used this mixture for both the sensitizing and intoxicating doses. In the first series of experiments guinea-pigs were injected intraperitoneally with a mixture of salvarsan and serum, and after four weeks were injected intravenously with a similar mixture. At the time of reinjection the pigs showed toxic symptoms resembling anaphylaxis, while controls injected with the same amount of the mixture or with salvarsan alone showed no symptoms at all. In subsequent experiments the controls of one day served as sensitized animals after a suitable lapse of time.

Table I shows a typical experiment. One of the guinea-pigs, B. 46, was sensitized by intravenous injection of salvarsan and guinea-pig serum, and on reinjection with a similar mixture after twenty-five days showed no symptoms. The other was first injected intraperitoneally, and after twenty-two days was reinjected intravenously. It showed immediate prostration, marked inspiratory dyspnea and death in ten minutes. The autopsy, performed immediately, showed the heart still beating. The lungs were much distended, filling the entire thoracic cavity. They had a leathery feel and on section were dry. This animal showed a typical anaphylactic death, with characteristic condition of the lungs described

7. Klausner, München. med. Wchnschr., 1910, lviii, 1451.

8. Friedberger, E., and Ito, T.: Ztschr. f. Immunitätsforsch., 1912, xii, 241.

9. Auer, J.: Jour. Exper. Med., 1911, xiv, 497.

by Auer and Lewis.¹⁰ At the beginning of the experiment the toxicity of salvarsan diluted with saline as well as that of a mixture of the same amount of salvarsan and serum were tested on normal guinea-pigs, and at the end of the experiment another control was injected with salvarsan and serum to prove that the mixture had not become toxic during the time of the experiment. This double con-

TABLE 1.

Experiment on the Toxicity of a Second Injection of a Mixture of Salvarsan and Guinea-Pig Serum.

First Injection.						Second Injection.							Symptoms.
Guinea-Pig.	Weight (Gm.).	Method.	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Stood (Min.).	Interval (Days).	Weight (Gm.).	Method.	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Stood (Min.).	
B. 46	300	Intraven.	5	3	70	25	300	Intraven.	5	3	.	40	Practically none. Prostration, dyspnea; died in ten min. Autopsy: distended lungs.
B. 54	250	Intraper.	5	3	80	22	240	Intraven.	5	3	.	15	
B. 62	Control					220	Intraven.	5	.	3	5	None.	
B. 64	Control					220	Intraven.	5	3	.	30	None.	
B. 65	Control					220	Intraven.	5	3	.	40	None.	

trol was used throughout the series, because it was found that the salvarsan rapidly becomes toxic on standing. It was found necessary to work with freshly prepared salvarsan solution and with fresh serum. The salvarsan was a 1 per cent. alkaline watery solution, always made with freshly distilled and sterilized water. The saline used in the controls was freshly prepared in the same way. The guinea-pig serum was obtained by bleeding the pigs in the usual way and separating the serum from the clot by centrifugalization. The serum was used within six hours of the time of bleeding, as it was found that old serum formed a more toxic mixture than fresh serum. This toxic effect with the first injection of controls was carefully watched and only those experiments in which it was not shown were accepted. It was found that guinea-pigs which weighed only 200 to 220 gm. were unsuitable, because a number

10. Auer, J., and Lewis, P. A.: Jour. Exper. Med., 1910, xii, 151.

showed toxic symptoms or death on first injection of 5 mg. of salvarsan mixed with guinea-pig serum. It will be seen in table 3 that all the animals which showed anaphylactic symptoms weighed 240 gm. or more.

TABLE 2.

Completely Controlled Experiment on Toxicity of Salvarsan-Serum Mixture.

First Injection—Intravenous.						Second Injection After Forty-Two Days—Intravenous.						
Guinea-Pig.	Weight (Gm.).	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Stood (Min.).	Symptoms.	Weight (Gm.).	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Stood (Min.).	Symptoms.
B. 13	320	5	2	...	15	Slight cramps.	290	5	3	...	37	Slight dyspnea; tremors, one hour; cramps, 30 minutes.
B. 26	290	5	2	...	15	None.	290	5	3	...	50	Jumping, 20 minutes; cramps, 1½ hours.
B. 14	270	5	2	...	23	None.	320	5	3	...	60	None.
B. 25	270	5	2	...	15	Moderate cramps.	220	5	3	0.5	5	Slight dyspnea, one-half hour.
B. 20	270	5	2	...	12	None.	290	5	3	...	50	None.
B. 27	240	3	1.5	...	18	None.	270	5	3	...	70	None.
B. 23	270	2	0.5	...	5	None.	320	5	3	...	40	None.
B. 44	Control	270	5	3	...	7	None.
B. 45	Control	270	5	3	...	18	None.
B. 46	Control	300	5	3	...	70	None.

Table 2 shows a completely controlled experiment. Two pigs previously injected with the salvarsan-serum mixture showed marked toxic symptoms on reinjection with a similar mixture, while controls which received at the same time either salvarsan or serum alone were free from symptoms. Also pigs which received a first injection of salvarsan alone showed no symptoms on reinjection with salvarsan and serum or salvarsan and saline. This experiment proves that it is necessary both to sensitize and to shock with a mixture of salvarsan and serum.

A complete list of the animals used in eight different experiments is given in table 3. In each of the experiments normal animals of the same or less weight than the sensitized animals of the day were used to control the toxicity of the salvarsan-serum mixture. Among nineteen sensitized animals three died acutely at the time of the second injection and had characteristic autopsy findings, two had

TABLE 3.
A Complete List of Animals Used in Eight Experiments.

Guinea-Pig.	Weight (Gm.).	Method.	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Symptoms.	Interval (Days).	Weight (Gm.).	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Stood (Min.).	Symptoms.
13	440	Intraper.	2.5	1	None.	28	500	11	1.1	45	Dyspnea: prostration; death in 2 hours. Autopsy: distended lungs; hemorrhages in stomach.
B. 1	400	Intraven.	10	1	None.	20	400	1	0.5	10	Dyspnea; prostration; death in 3 hours. Autopsy: distended lungs.
B. 54	250	Intraper.	5	3	None.	22	240	5	3	15	Dyspnea; prostration; death in 10 min. Autopsy: distended lungs.
14	440	{ Intraper. Intraven.	2.5 10	1 3	None. Cramps.	50 19	440 550	5 5	2 3	10	Dyspnea; prostration; death in 10 min. Autopsy: distended lungs.
16	440	Intraper.	20	1	None.	29	550	7.5	3	45	Prostration; dyspnea. Recovery.
12	500	Intraper.	1	1	None.	29	490	10	3	50	Prostration. Recovery.
14	440	Intraper.	2.5	1	None.	29	440	10	3	40	Cramps; sneezing. Recovery.
B. 13	320	Intraven.	5	2	Slight cramp, 10 minutes.	42	290	5	3	5	Slight dyspnea; tremors 1 hour; cramps 30 minutes. Recovery.
B. 26	290	Intraven.	5	2	None.	42	290	5	3	37	Jumping 20 minutes; cramps 1 1/4 hours. Recovery.
B. 3	400	Intraven.	10	3	None.	19	400	5	2	5	Slight cramps one-half hour. Recovery.
B. 5	400	Intraven.	10	3	Cramps.	13	400	5	2	15	Slight prostration 25 minutes. Recovery.
B. 6	350	Intraven.	5	3	Slight cramp.	24	420	5	2.5	30	Dyspnea 15 minutes. Recovery.
B. 64	220	Intraven.	5	3	None.	21	200	5	3	45	None.
B. 4	350	Intraven.	10	3	None.	19	350	5	2	15	None.
B. 46	300	Intraven.	5	3	None.	25	300	5	3	40	None.
12	500	{ Intraper. Intraven.	1 7.5	1 3	None. Prostration; rapid recovery.	32 13	490 490	5 5	2 2	10	None.
B. 7	500	Intraven.	5	3	None.	24	520	5	2.5	22	None.
B. 8	350	Intraven.	5	3	None.	24	370	5	3	40	None.
B. 68	200	Intraper.	5	3	None.	21	240	4	2.4	70	None.

TABLE 3.—Continued.

Guinea-Pig.	Weight (Gm.).	Method.	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Symptoms.	Interval (Days).	Weight (Gm.).	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Stood (Min.).	Symptoms.
CONTROLS.													
B. 14	270	Intraven.	5	2	None.	40	320	5	...	3	60	None.
B. 17	540	Intraper.	20	.	2	None.	29	540	5	3	10	None.
B. 62	220	Intraven.	5	.	3	None.	21	300	5	3	40	None.
B. 20	270	Intraven.	5	.	2	None.	42	290	5	3	50	None.
B. 27	240	Intraven.	3	2	1.5	None.	42	270	5	...	3	70	None.
B. 23	270	Intraven.	2	0.5	None.	42	320	5	3	40	None.
B. 11	400	Intraper.	1	Cramps; moderate.	29	420	5	3	30	None.
B. 25	270	Intraven.	5	2		42	220	3	0.5	5	Dyspnea, slight, one-half hour.

very marked symptoms, four showed marked symptoms, three had slight symptoms and seven showed no symptoms on reinjection. The first series of eight controls demonstrates that it is necessary to use both salvarsan and serum at both injections. Seventeen normal controls gave no evidence of intoxication with the first salvarsan injection, and in seven other normal animals the salvarsan when diluted with saline was not toxic.

This series of experiments demonstrates a very interesting effect of salvarsan. An animal never shows an anaphylactic reaction with untreated homologous serum. The salvarsan alone in the dose employed is not toxic for the guinea-pigs, nor does a single injection of salvarsan render a guinea-pig hypersensitive to a subsequent injection. Nevertheless, the mixture of salvarsan and serum, which on first injection is non-toxic, so alters the condition of the animal that a second injection of the same mixture is toxic. It seems that the action of the salvarsan on the serum changes the nature of the serum so that it acts like a foreign proteid and induces the condition of hypersensitiveness. This is analogous to the alteration in a homologous serum by iodids, which was demonstrated by Friedberger and Ito. In our series of experiments, however, the anaphylactic condition was not induced by the pure chemical substance alone. The possibility is suggested that in mixtures of chemical substances and blood-serum, combinations may occur which alter the nature of the protein in such a way that when injected into animals of the same species from which it was derived, it may react in the manner of a foreign protein. Whether such combinations occur *in vivo* on first administration of a drug and so induce the sensitization of the individual is a matter of considerable interest in the study of drug idiosyncrasies.

In the patients who showed anaphylactic-like symptoms after repeated injections, some such reaction may have occurred. When introduced into the blood-stream, a combination of salvarsan takes place with the serum. Repeated injections and combinations of this nature probably cause the body to immunize itself against this denaturized serum, so that on a fourth or subsequent injection sufficient alteration in the reactive powers has been brought about to produce the peculiar set of symptoms. The hypersensitive con-

dition developed by patients after repeated injections of salvarsan passes off after a certain period of time. In one patient, however, this condition was more easily induced and the symptoms were more severe with a second series of injections than with the first. This is an example of so-called accelerated reaction. In another patient the hypersensitive condition persisted for more than eight months. Whether moderate-sized doses of salvarsan predispose more to the development of the condition than large doses is still an open question. The condition is one which is relatively infrequent, and when it occurs, if its nature is recognized, need not necessarily be serious. The immediate treatment during the attack is to discontinue the injection. At times the injection may be renewed after ten minutes without a return of the symptoms. This insusceptibility may be explained as a condition of anti-anaphylaxis. If subsequent injections are necessary, our experience with two cases seems to indicate that neosalvarsan may be substituted for salvarsan. After a certain lapse of time the hypersensitiveness usually disappears and then treatment may be renewed.

Unsensitized Controls.

Guinea-Pig.	Weight (Gm.).	Salvarsan (Mg.).	Guinea-Pig Serum (C.c.).	Saline (C.c.).	Stood at 37° (Min.).	Symptoms.
B. 1	400	10	1	...	60	None.
B. 36	290	5	2	...	18	None.
B. 8	350	5	3	...	25	None.
B. 6	350	5	3	...	15	None.
B. 45	270	5	3	...	18	None.
B. 46	300	5	3	...	70	None.
B. 64	220	5	3	...	30	None.
B. 65	220	5	3	...	40	None.
B. 30	350	5	2	...	5	None.
B. 31	250	5	2	...	20	None.
B. 33	290	10	2	...	5	None.
B. 34	320	10	2	...	15	None.
B. 4	356	10	3	...	25	None.
B. 5	400	10	3	...	65	None.
B. 42	250	5	2.5	...	10	None.
B. 43	290	5	3	...	60	None.
B. 78	270	4.5	2.7	...	30	None.
B. 37	350	5	...	2	60	None.
B. 44	270	5	...	3	7	None.
B. 62	220	5	...	3	5	None.
B. 32	300	5	...	2	5	None.
B. 3	400	10	...	3	40	None.
B. 41	300	5	...	3	10	None.
B. 77	270	4.5	...	2.7	20	None.

SUMMARY.

After repeated injections of salvarsan, certain patients show symptoms of a respiratory and vasomotor nature like those seen in anaphylaxis, and in one of our cases there occurred a toxic erythema. Guinea-pigs which have been sensitized by the injection of a mixture of guinea-pig serum and salvarsan, and have been reinjected, after a suitable time, with the same mixture, show symptoms like those seen in anaphylactic shock. This phenomenon seems to depend on an alteration of the native serum by salvarsan so that the homologous serum acts like a foreign proteid. In patients who show anaphylactic symptoms on repeated injections of salvarsan, a similar reaction probably takes place between the patient's own serum and salvarsan.

SECONDARY SYPHILITIC MENINGITIS.*

By ARTHUR W. M. ELLIS, M.B.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

The subject of syphilitic meningitis, occurring early in the secondary period of the disease, is one which has attracted the attention of occasional observers since 1650, when Guarinoni¹ first described the condition. The meningitis usually shows itself first as a disturbance of sight or hearing and may go on to the typical picture of an acute or subacute diffuse meningitis. In Germany, Rumpf,² Knorre,³ Lang⁴ and Nonne,⁵ in France, Gros and Lancereaux,⁶ Ravaut,⁷ Fournier,⁸ Boidin and Weil,⁹ and in England, Mott¹⁰ and Williamson¹¹ have especially called attention to the condition. The advent of salvarsan has, however, given the question a prominence which it never possessed before and has led to a vigorous discussion of the real frequency of its occurrence and of the possibility of salvarsan as a predisposing factor.

In November, 1910, Finger¹² in Vienna, in his report on 170

* Read in the Symposium on Syphilis in the Section on Genito-Urinary Diseases of the American Medical Association, at the Sixty-Third Annual Session, held at Atlantic City, June, 1912.

1. Guarinoni: Cited by Benario, Ueber Neurorezidive, Munich, 1911.
2. Rumpf: Die syphilitischen Erkrankungen des Nervensystems, J. F. Bergmann, Wiesbaden, 1887.
3. Knorre: Deutsch. Klin., 1849, Nos. 6 and 7.
4. Lang: Vrtljschr. f. Dermat. u. Syph., 1881, p. 469.
5. Nonne: Syphilis und Nervensystem, S. Karger, Berlin, 1909.
6. Gros and Lancereaux. Des affections nerveuses syphilitiques, Paris, 1861.
7. Ravaut: Ann. de dermat. et syph., 1903, p. 537.
8. Fournier: La syphilis du cerveau, Paris, 1879.
9. Boidin and Weil: Presse méd., 1907, p. 681.
10. Mott: System of Syphilis, Vol. IV., London, 1910.
11. Williamson: Syphilis and Syphilitic Diseases of the Spinal Cord, Sherrett and Hughes, Manchester, 1899.
12. Finger: Wien. klin. Wchnschr., 1910, xxiii, 1667.

cases of syphilis treated with salvarsan, reported seven cases showing affections of the optic and auditory nerves coming on a few hours to some months after treatment. He considered them examples of a neurotropic action of the drug. In a later communication by Mucha¹³ from the same clinic, thirty-seven similar cases were reported. The contentions of Finger immediately aroused widespread discussion, and in the past year a very large number of these cases of affections of the cranial nerves after salvarsan have been reported.

Ehrlich,¹⁴ from the beginning, held that these affections of the cranial nerves were due, not to a toxic action of salvarsan, but to a syphilitic meningitis developing from foci of spirochetes in the central nervous system, which had not been reached by the salvarsan. He gave them the name "neurorecidiv," and considered that the increased frequency of such relapses was due merely to a closer observation and therefore more frequent recognition.

Benario,¹⁵ in his collected statistics, showed the frequency of such nerve relapses in mercury-treated patients, and pointed out the exact similarity of such cases to the nerve disturbances following treatment with salvarsan. This similarity and the findings obtained by lumbar puncture have proved the correctness of Ehrlich's opinion as to the syphilitic nature of the process. Lumbar puncture in these cases has shown, almost invariably, large increase in the cells, increased globulin and a positive Wassermann reaction in the spinal fluid.

The question of the relative frequency of early secondary meningitis with and without salvarsan therapy still remains an open one.

The occurrence of these nerve relapses after salvarsan is, in the experience of most observers, from 0.5 to 3 per cent. The figures of Finger, who has seen forty-four nerve relapses in 500 cases treated, remain unparalleled. The following tabulation gives the results in some of the large clinics:

Author.	No. of cases treated.	No. of nerve relapses.
Desneux and Dujardin ¹⁶	350	7
Rille ¹⁷	200	3

13. Mucha: *Wien. klin. Wchnschr.*, 1911, xxiv, 1012.

14. Ehrlich: *München. med. Wchnschr.*, 1911, lviii, 2481.

15. Benario: *Ueber Neurorezidive*, Munich, 1911.

16. Desneux and Dujardin: *München. med. Wchnschr.*, 1911, lviii, 1245.

17. Rille: *Berl. klin. Wchnschr.*, 1910, xlix, 2281.

Author.	No. of cases treated.	No. of nerve relapses.
Krausz ¹⁸	345	5
Von Zeissl ¹⁹	273	4
Frühauf ¹⁸	478	3
Gennerich ²⁰	340	1
Krausz ¹⁸	345	5
Simonyi ²¹	300	0
Gibbard, Harrison and Kane ²² ..	392	1
Neisser ²³	2,000	6
Scholtz ¹⁸	700	3
Klingmüller ¹⁸	1,260	4
Schwarz ²⁴	*	5
Gérone and Gutmann ²⁵	300	13
Milian ²⁶	1,200	5
Finger	500	44

* Not mentioned.

Such figures show undoubtedly that syphilitic meningitis is much more frequent than we have considered it in the past. Two possibilities arise: (1) that more careful observation has led to more frequent recognition; (2) that salvarsan in some way predisposes to the development of syphilis of the nervous system.

Numerous observers have called attention to the frequency of involvement of the nervous system in the early stages of syphilis. Jarisch²⁷ noticed the increased reflexes at this period. Fournier described a regional analgesia; Finger²⁸ himself, in 1881, called attention to the increased irritability of the reflexes during the period of the exanthem, followed by diminished excitability and, in some cases, by disappearance.

Lang,⁴ in 1881, referred to the frequent headache, neuralgia, and dizziness occurring in the secondary stage and suggested that they

18. Cited by Benario: *Wien. med. Wchnschr.*, 1912, lxii, 583.

19. Von Zeissl: *Berl. klin. Wchnschr.*, 1911, xlviii, 1785.

20. Gennerich: 3. Bericht über Salvarsanbehandlung. S. 12, Berlin, 1911.

21. Simonyi: Cited by Benario. (See Note 18).

22. Gibbard, Harrison and Kane: *Jour. Royal Army Med. Corps*, January, 1912.

23. Neisser: *Ueber moderne Syphilistherapie*, Halle a. S. 1911.

24. Schwarz: *St. Petersburg med. Ztschr.*, 1912, xxxvii, 99.

25. Gérone and Gutmann: *Berl. klin. Wchnschr.*, 1911, xlviii, 416.

26. Milian: *Bull. et mém. de la Soc. méd. d. Hôp. de Paris*, 1912, xxviii, 326.

27. Jarisch: Cited by Mott. (See 10).

28. Finger: *Ueber eine constante nervöse Störung bei florider Syphilis der secundärperiode*, *Vrtljschr. f. dermat. u. syph.*, 1881, p. 255.

were signs of meningeal irritation. Schnabel²⁹ in the same clinic examined the eyes of forty patients in the secondary stage of syphilis and found in seven retinitis, chorioiditis or chorioretinitis; fourteen others showed some degree of retinal hyperemia.

Wilbrandt,³⁰ in Hamburg, examined the eye-grounds of 200 syphilitics in the early stages of the disease and found hyperemia thirty-eight times, neuroretinitis five times, retinitis once and retinal hemorrhage once. The frequency of disease of the internal ear in the early stages of syphilis has been noted by several authors.

Habermann,³¹ in 1896, reported sixty-six cases of paralysis of the acoustic nerve due to syphilis, thirty-four of which appeared in the secondary stage of the disease. He pointed out the early period at which such lesions might appear. Of the thirty-four occurring in the secondary stage, three appeared on the day of the eruption and in only six was the disturbance of hearing first detected later than ten weeks after the exanthem. Mayer³² in the same clinic has seen sixty-five examples of acoustic paralysis due to syphilis, of which thirty occurred in the first year.

Frey³³ has collected a large number of reports of cases of syphilitic disease of the middle ear from the literature. He considers the increased frequency of such lesions after salvarsan by no means proved.

The most important contribution to the subject of early involvement of the central nervous system in syphilis was made by Ravaut,³⁴ in 1903. He examined the spinal fluid in 116 cases of secondary syphilis and found some degree of abnormality, either increased cells or increased protein content, in 67 per cent. In a subsequent paper he reports the examination of thirty-three patients in the secondary stage treated with salvarsan; in them he found abnormality of the spinal fluid in 85 per cent. He considers, therefore, that salvarsan increases the frequency of meningeal involvement. Both papers are open to criticism in that the methods employed in examination are

29. Schnabel: *Vrtljschr. f. dermat. u. syph.*, 1881, p. 469.

30. Wilbrandt: Cited by Schwarz. (See 24).

31. Habermann: *Die luetischen Erkrankungen des Gehörorgans*, Jena, 1896.

32. Mayer: *Wien. klin. Wchnschr.*, 1911, xxiv, 381.

33. Frey: *Wien. klin. Wchnschr.*, 1911, xxiv, 385.

34. Ravaut: *Presse méd.*, 1912, No. 18. (Also see 7).

not the most accurate, and in that Ravaut counts as abnormalities changes which the majority of authorities do not accept as being necessarily pathologic. The changes in many of the cases are sufficient, however, to show how frequently syphilitic involvement of the meninges does occur in the secondary period.

The degenerations of cranial nerves following the use of atoxyl, arsacetin and other organic arsenic preparations impressed people with a fear of a similar action for salvarsan. This fear was really unjustified because the chemical structure of salvarsan differs entirely from the earlier preparations. In these the arsenic was in the pentavalent form, while in salvarsan it is trivalent. Moreover, the phenyl-arsenic acid group, which Igersheimer has shown to be responsible for the neurotoxic action of atoxyl and similar compounds, is not present in salvarsan. This fear of a neurotoxic action of salvarsan led to a very careful examination and careful following up of patients treated with the drug, and resulted in the ophthalmologists and otologists seeing many more cases of early syphilis than ever before. In this lies in part, at least, the increased frequency of disturbances in sight and hearing in the early stages of syphilis noticed since the introduction of salvarsan. In this connection the figures of Werther³⁵ are interesting. Werther, in the past year, has seen twelve cases of meningitis in early secondary syphilis. Three of these had had no treatment, four had had mercury, and five had been treated with salvarsan. Similarly, Kren¹⁸ reports having seen in one month two cases of meningitis in patients treated with mercury.

The supporters of the theory of an increased susceptibility of the nervous system due to salvarsan have advanced several hypotheses, none of which seems very acceptable. Finger considers that salvarsan injures the walls of the small cerebral vessels, leading to a *locus minoris resistentiæ* for syphilitic arteritis. This subsequently spreads and involves the nervous tissue. Other authors consider that salvarsan changes the reaction of the body to the syphilitic virus and use as a simile the occurrence of the so-called Thallmann's chancre on the skin. Others again consider that there is "an abolition of inhibiting substances" or "an increased supply of the food

35. Werther: München. med. Wchnschr., 1911, lviii, 505.

material necessary for growth." Ehrlich¹⁴ has suggested that the sterilization of the rest of the body leads to increased development of the few spirochetes remaining in the central nervous system, inaccessible to salvarsan. He has used as an illustration the growth of bacteria occurring on culture plates. If many bacteria are planted, large numbers of very small colonies appear; if only a few bacteria are introduced, few colonies of large size occur.

At the hospital of the Rockefeller Institute we have seen during the past nineteen months six cases of secondary syphilitic meningitis. All of these patients had had salvarsan. Four cases occurred in our own series of salvarsan-treated patients, which contained thirty-two in the secondary stage of the disease, all but one of whom have now been followed for at least one year. At first sight the frequency of our cases of syphilitic meningitis, four in thirty-two secondary cases treated, appears as great as the figures given by Finger. Only one of the four patients, however, presents the typical picture of a nerve recurrence with paralyses of cranial nerves. In the other three cases, the signs of involvement of the nervous system were quite indefinite and the diagnosis could not have been made without the assistance of lumbar puncture. In addition to these four cases, we have had an opportunity of studying two patients (Cases 5 and 6), both typical examples of the so-called nerve recurrences, occurring in the practice of others, and referred to us for treatment.

Case 1.—May 17, 1911: Chancre of the left index-finger. June 30, 1911: secondaries. Wassermann reaction ++. July 8: 0.5 gm. salvarsan intravenously. July 20: 0.2 gm. salvarsan. August: five injections of mercury salicylate. September 6: ringing in the ears, slight dizziness for one day; Wassermann reaction negative. September 9: 0.4 gm. salvarsan. October and November: five injections of mercury salicylate. November 24: vertigo, nausea, vomiting, ringing in the ears, deafness; stiffness of the muscles of the neck. December 8: unequal pupils, papillitis, nystagmus, right ear diminished air conduction, complete loss of bone conduction and facial paresis; Wassermann reaction negative. Lumbar puncture: fluid hazy, cell-count 575, Noguchi globulin ++. Wassermann reaction in spinal fluid 0.5 c.c. +. December 9: 0.4 gm. salvarsan. December 17: hearing improved, nausea and vertigo have disappeared. May 6, 1912: mercury to point of tolerance; patient feeling well; hearing improved; occasional tinnitus still present; Wassermann reaction negative.

In this patient the first symptom suggesting involvement of the nervous system appeared two and a half months after the secondary

eruption, patient having had 0.7 gm. salvarsan and four mercury injections. Improvement under further salvarsan therapy was rapid.

Case 2.—December 27, 1910: primary. February 7, 1911: secondaries of malignant type, headache, lancinating pain in the right ear, pains in the legs. Mercury by mouth for one month. April: severe pain about the right eye, dimness of vision, spots before the eyes, intense headache, tenderness of the scalp. Twenty inunctions of mercury. May 5, 1911: inequality of pupils, exaggerated reflexes; Wassermann reaction negative. May 8: 0.4 gm. salvarsan. May: four injections of mercury salicylate. June 10: 0.4 gm. salvarsan. August 1: general malaise, inability to work, headache, tenderness of the scalp, itching sensation over whole body. Five injections of 0.2 gm. salvarsan; complete disappearance of symptoms. October 4: headache, stabbing pain in left ear, numbness of the left side of the body; Wassermann reaction negative. October 6: lumbar puncture; cell-count 132, Noguchi globulin +, Wassermann reaction in spinal fluid, 0.1 c.c. —. October 7 to October 20: three injections of 0.2 gm. salvarsan. October 25 to October 30: dazed hysterical mental condition. November: two injections of 0.2 gm. salvarsan. November 18: lumbar puncture; cell-count 173, Noguchi globulin +, Wassermann reaction in spinal fluid, 0.4 c.c. —. May 23, 1912: has been taking mercury by mouth and has felt perfectly well since the beginning of January. Reflexes still exaggerated. Lumbar puncture refused.

In this case the infection of the meninges almost certainly dates from the time of appearance of the secondary rash, and before any salvarsan had been administered. The headache, dimness of vision, spots before the eyes, lancinating neuralgic pain and exaggerated reflexes occurring at this time all indicate the probability of a meningitis being already present.

Case 3.—July, 1911: sore throat, probably chancre of the tonsil, headache, 1911, to January 1, 1912: nine injections of 0.2 gm. and one of 0.3 gm. salvarsan. October 10: admission to the hospital; Wassermann reaction ++. October 1, 1911, to January 1, 1912: nine injections of 0.2 gm. and one of 0.3 gm. salvarsan. Marked improvement, mental depression has disappeared, headache much less frequent. Discharged. January 1, 1912, to March 1, 1912: four mercury injections and mercury by mouth for two weeks. March 4, 1912: readmission to hospital; mental depression, general malaise, one small area of skin hyperesthesia; knee-jerks exaggerated. Lumbar puncture: cell-count 110, Noguchi globulin ±, Wassermann reaction in spinal fluid, 0.5 c.c. ++. March 4 to April 5: five injections of 0.3 gm. salvarsan. Improvement; disappearance of mental depression, no headache; lumbar puncture: cell-count 14, Noguchi globulin —, Wassermann reaction in spinal fluid, 0.5 c.c. —. April 5 to May 25: four injections of 0.3 gm. salvarsan and two injections of mercury. Feeling well, knee-jerks still very active.

In this patient, the signs of involvement of the nervous system

were of the most trivial character, yet lumbar puncture showed an active syphilitic meningitis to be present. Headache, mental depression and exaggerated knee-jerks were the only symptoms at any time. It is significant that these same symptoms were present even before the appearance of the secondary exanthem and before any treatment had been administered.

Case 4.—October 15, 1910: primary. November 15: malaise, pains in the shoulders and in the ankles, indistinctness and blurring of vision. November 22: secondary rash. November 26: 0.6 gm. salvarsan intramuscularly. December 1: patient feeling well. December 30: complains again of "filminess" before the eyes and pain in the right ankle, stiffness of the muscles of the neck and some tenderness over both olecranon processes. January 9, 1911: 0.5 gm. salvarsan intravenously. January 23: still has occasional attacks of filminess before the eyes. February 1 to April 1: six injections of mercury salicylate and eleven of enesol. April 21: general papular eruption, mucous patches, pains in ankles and elbows. May 10: 0.4 gm. salvarsan. June 19: Wassermann reaction negative. June 1 to August 15: ten injections of mercury salicylate and eight of mercuric chlorid. August 15: Wassermann reaction ++. September 15: mucous patches. September 29 to October 27: five injections of 0.2 gm. salvarsan. November 27: mucous patches, palmar and plantar papular syphilids, Wassermann reaction ++. December 22: itching sensation in the axillæ and down the sides of both thighs, blepharospasm, pain about the left ankle. December 1 to December 29: four injections of 0.3 gm. salvarsan. January 1, 1912, to March 1: eight injections of mercury. March 5: hyperesthesia of both knees lasting two days. Patient noticed that his overcoat flapping against his knees hurt him. March 1 to March 29: five injections of 0.3 gm. salvarsan. March 29 to May 23: eight injections of mercury. May 23: lumbar puncture: cell-count 212, globulin \pm , Wassermann reaction in spinal fluid, 0.4 c.c. ++. Wassermann reaction in the blood negative.

This is the most resistant case of syphilis we have seen. As soon as treatment was discontinued, the patient immediately relapsed and after eighteen months of almost continuous treatment with mercury and salvarsan, he still has what is evidently an active meningitis. On reading back through the history, one is struck with the occasional headaches, skin hyperesthesia, paresthesia, blepharospasm, pains in the legs and filminess of vision, all symptoms probably referable to the nervous involvement and appearing at intervals from the beginning of the infection. Here again symptoms were noticed before the beginning of treatment which, in the light of subsequent knowledge, suggest involvement of the meninges, even at this early date.

TABLE OF CASES OF SECONDARY SYPHILITIC MENINGITIS.

Case No.	1	2	3	4	5	6
Primary.....	5/17/11 6/30/11	12/27/10 2/7/11	Not known..... 6/15/11	10/15/10 11/22/10	5/1/11 5/20/11	10/18/11
Secondaries.....	Deafness, dizziness for one in the ear, ten-day. 9/10/11.	Headache, pain in the ear, tenderness for one in the ear, ten-day. 2/9/11.	Headache. 6/11.	Dimness of vision. 11/20/10.	Headache, deafness, facial paralysis. 8/19/11.	Headache, tender scalp. 11/15/11
Treatment before first nervous symptom.....	0.7 gm. salvarsan. 4 Hg injections.	Hg by mouth for 2 weeks.	None.....	None.....	1.5 gm. salvarsan. 6 Hg injections. 12 Hg injections. 9/23/11	None.
Date of first lumbar puncture.....	12/8/11	10/4/11	3/7/12	5/23/12	4/16/12	4/16/12
Cell-count in spinal fluid.....	575	132	110	212	1074	190
Globulin in spinal fluid.....	++	+	+	+	++	+
Wassermann reaction in spinal fluid.....	0.5 c.c. +	0.1 c.c. —*	0.5 c.c. ++	0.4 c.c. ++	0.1 c.c. ++	0.4 c.c. ++
Wassermann reaction in blood at time of first lumbar puncture.....	—	—	—	—	++	+
Treatment before first lumbar puncture.....	1.1 gm. salvarsan. 8 Hg injections.	1.8 gm. salvarsan. 4 Hg injections.	2.8 gm. salvarsan. 4 Hg injections.	5.4 gm. salvarsan. 33 Hg injections. 11 injections enesol.	Same as above and 2 weeks' mixed treatment.	1.0 gm. salvarsan.
Date of last lumbar puncture.....	Lumbar puncture refused.	Lumbar puncture refused.	6/11/12	First lumbar puncture only 2 weeks ago.	5/31/12	5/22/12
Cell-count in spinal fluid.....	8	8	23
Globulin in spinal fluid.....	—	—	—
Wassermann reaction in spinal fluid.....	0.5 c.c. —	0.5 c.c. —	0.5 c.c. ++
Wassermann reaction in blood at time of writing.....	—	—	++
Treatment between first and last lumbar puncture.....	3.0 gm. salvarsan. 3 Hg injections.	5.5 gm. salvarsan. 9 Hg injections.	0.9 gm. salvarsan.

* Larger amounts of fluid not used at this date.

In the above table the date of first lumbar puncture marks the time at which definite suspicion of nervous involvement first arose, except in Cases 5 and 6, which were referred to us on account of their nervous symptoms and were lumbar punctured on admission.

Case 5.—May 1, 1911: primary. May 29: secondaries. June 9 to June 24: three injections of 0.5 gm. salvarsan. July 1 to August 15: six injections and twelve injections of mercury. August 19: stiffness of the muscles of the neck, lancinating headache, deafness in the left ear, dizziness, weakness of the left side of the mouth. September 23: admission to the hospital; unequal pupils, exaggerated reflexes. Lumbar puncture: fluid hazy, cell-count 1,074, Noguchi globulin ++, Wassermann reaction in spinal fluid, 0.1 c.c. ++; Wassermann reaction in serum ++. September 28: 0.2 gm. salvarsan; immediate improvement. October 5: 0.2 gm. salvarsan; improvement continues. October 12: 0.2 gm. salvarsan. October 16: feels perfectly well, hearing in left ear only slightly impaired. Lumbar puncture: cell-count 118, Noguchi globulin +, Wassermann reaction in spinal fluid, 0.1 c.c. —. October 12 to November 29: seven injections of 0.2 gm. salvarsan. Wassermann reaction in blood +±. December 5: lumbar puncture: cell count 60, Noguchi globulin +, Wassermann reaction in spinal fluid, 0.5 c.c. +. December 7 to December 14: two injections of 0.2 gm. salvarsan. January, 1912: five injections of mercury salicylate. January 21: lumbar puncture: cell-count 9. February: four injections of mercury. March 1: general malaise, pains in arms and legs. Lumbar puncture: cell-count 46, Noguchi globulin +, Wassermann reaction in spinal fluid, 0.5 c.c. ± (?). March 9 to April 16: five injections of 0.3 gm. salvarsan and one of 0.4 gm. Wassermann reaction negative. April 18: lumbar puncture: cell count 11, Noguchi globulin ±, Wassermann reaction in spinal fluid, 0.5 c.c. —. May 7 to May 28: four injections of 0.3 gm. salvarsan. May 31: lumbar puncture: cell-count 8, Noguchi globulin —, Wassermann reaction in spinal fluid 0.5 c.c. —.

This is a typical example of the so-called nerve recurrences, paralysees of cranial nerves coming on three months after infection in a patient treated with salvarsan. In this case no sign of involvement of the nervous system can be made out in the earlier history. The curative effect of continued, intense salvarsan therapy in such cases of meningitis is well demonstrated. The patient has had 7 gm. of salvarsan in the course of the year.

Case 6.—October 18, 1911: primary. No secondaries. November 15: severe frontal and vertical headache, pain in the eyes, tenderness of the scalp. November 28: 0.5 gm. salvarsan; headache disappeared. December 25: headache severe, deafness in the right ear. January 22, 1912: 0.5 gm. salvarsan; gradual improvement. April 1: headache returning. April 11: admitted to hospital; pupils irregular, reflexes exaggerated; Wassermann reaction +. Lumbar puncture: cell-count 190, Noguchi globulin —±, Wassermann reaction in spinal fluid, 0.4 c.c. ++.

The severe vertical and frontal headache, tenderness of the scalp and pain in the eyes before any treatment was given again suggest that the meningitis was then already present, in this case, even before the appearance of secondaries.

A review of these cases is instructive. In four of the six, symptoms referable to the nervous system were already present before salvarsan had been given, in two cases even before the appearance of secondaries. In four of the cases, although the indications point to an active meningitis having been present over a period of months, the symptoms were never obtrusive and only lumbar puncture revealed the nature of the process. The triviality of the symptoms which may accompany syphilitic infection of the meninges has been called attention to by Schwarz, who noticed in several cases of nerve relapses, with improvement under treatment, complete euphoria, although examination of the spinal fluid showed that a high cell count and positive Wassermann reaction in the spinal fluid was still present. Hoffmann³⁶ has been able to produce a primary lesion in a monkey by inoculation of the spinal fluid of a case of early secondary syphilis, absolutely free from nervous symptoms. The beneficial result of continued, intense salvarsan therapy, which has been noticed by all observers, is well illustrated in Cases 3, 5 and 6.

Syphilitic secondary meningitis, therefore, may occur early in the disease. It may be latent, causing no obtrusive symptoms, over long periods. It is a more frequent complication than we have commonly considered. The so-called nerve recurrences after salvarsan are examples of such a meningitis. In most cases the infection of the nervous system has probably already occurred before the institution of treatment. They represent the development of the disease in a region notoriously difficult to reach with curative agents. The contention that salvarsan predisposes in any way to the development of disease of the nervous system has not yet been established.

36. Hoffmann: *Dermat. Ztschr.*, 1906, xiii, 561.

TOXIC SUBSTANCES PRODUCED BY PNEUMOCOCCUS.*

By RUFUS COLE, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

There is much obscurity concerning the relation between the growth of pneumococci in the body and the symptoms and death induced in animals and in man. The view that bacteria act mechanically was long ago held to be inadequate and incorrect. The discovery of toxic substances produced by the growth of certain bacteria outside the body, notably by *Bacillus diphtheriæ* and *Bacillus tetani*, gave support to the supposition that the effects of the growth of all bacteria in the body were due to poisons arising through the metabolic activities of the cells. Since the poisons, however, could not be demonstrated in the culture fluids of other bacteria, this supposition has not received final proof. The demonstration by Pfeiffer that certain bacteria killed by heat or other harmful agents are toxic, gave support to the view that the effects produced in the body by the growth of bacteria are not due to the products of growth of the bacteria, but to toxic substances set free upon their death and dissolution. The attempt to demonstrate these so called endotoxic substances in a large number of bacteria, however, has not been successful, and in the cases where they have been demonstrated, the exact relationship of the poisons to the symptoms and death induced has not yet been rendered perfectly clear.

These theories, however, do not exhaust the conceivable ways in which the growth of bacteria within the body may exert harmful effects, and it is possible that the effects may not be due to substances which may be isolated, or even be detected by injection into the animal body.

Nevertheless, at the present time, the only hope of arriving more closely at a conception of the process seems to be in the iso-

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lation, from the bacteria or their cultures, of substances having a similar effect to that of the living bacteria themselves.

Thinking that possibly such substances might be present in excess in the fluids of the infected animal, a series of experiments, of which the following is typical, were undertaken.

A rabbit was inoculated with an overwhelming dose of virulent pneumococci. Just before death, the animal was bled to as near complete exsanguination as possible, the serum was removed and passed through a Berkefeld filter in order to remove the bacteria, and as quickly as possible this filtered serum was injected into the veins of a normal animal.

The following is a protocol of one of these experiments:

Rabbit 7.—Weight 2,870 gm. Temperature 39° C.

Feb. 9, 1912, 12:05 P.M. Injected intravenously an emulsion in salt solution of the bacteria obtained by centrifugalization from 1,000 c.c. of a 24 hour bouillon culture of pneumococcus 1.4².

12:15 P.M. Bowels move actively. 1:10 P.M. Temperature 40° C. 2:10 P.M. Temperature 39.8° C. 3:10 P.M. Temperature 39° C. 4:10 P.M. Temperature 38.4° C. 5:00 P.M. Animal moribund. Animal bled as completely as possible. Blood allowed to clot, serum obtained by centrifugalization. Serum passed through Berkefeld filter under pressure.

Rabbit 8.—Weight 1,600 gm. Temperature 38.2° C.

Feb. 9, 6:30 P.M. Injected into ear vein filtered serum obtained from rabbit 7.

10:30 P.M. Temperature 39.6° C. Animal shows no symptoms.

Feb. 10, 10:00 A.M. Temperature 39° C. Weight 1,600 gm. Animal appears well. 6:00 P.M. Temperature 39° C.

Feb. 11, 10:00 A.M. Weight 1,620 gm.

During the succeeding days a record was kept of the weight. It lost slightly, weighing on February 24 1,510 gm. No other changes were noted.

Other similar experiments were conducted on a series of rabbits, but in no case were acute toxic symptoms noted, nor were chronic changes, as determined by loss of weight, observed. It therefore became evident that if the symptoms of infection by pneumococcus are due to soluble toxins formed in the serum of the infected animal, they are fixed by the tissues as rapidly as formed, or are extremely labile and so lose their toxicity on removal from the body, or possibly are held back with certain of the proteins during filtration.

Attention was then directed to the production of toxic substances by the action of immune serum and complement on pneumococci.

Friedberger,¹ in his study of the nature of anaphylaxis, has shown that by

¹Friedberger, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 692; 1909-10, iv, 636.

the action of complement on the precipitate formed by the addition of immune serum to the serum antigen, a toxic substance is formed which, on injection into a guinea pig, produces acute symptoms and death, apparently identical with the symptoms and death occurring in anaphylaxis due to the second injection of egg albumin or serum. To the toxic substance contained in the albumin-immune-serum-complement mixture, Friedberger gave the name anaphylatoxin. He considered that this experiment gave the final proof of the antibody reaction in anaphylaxis. Later he³ showed that a similar toxic substance appeared when bacteria were treated with immune serum and complement outside the body. This "Bakterien-anaphylatoxin" he considered identical with the anaphylatoxin produced from serum and identical with the substances formed within the body on the second injection of protein, to which hypothetical substances he considers the symptoms due. Friedberger thinks the symptoms in infection are due to this substance set free in the body. He defines infection as a "mild, protracted form of anaphylaxis" and anaphylaxis as an "extreme, acute form of infection." Therefore it is not necessary to assume any specific bacterial toxin in any infection, but Friedberger thinks in all infections the symptoms are induced by the same poisonous substance, and the different manifestations of the different infections depend only on different modes of infection, localization, etc., of the different microorganisms.

Neufeld and Dold⁴ were able to repeat the experiment of Friedberger, using pneumococcus as antigen. We have also repeated this experiment with pneumococcus. Since no essential differences in the results from those of Neufeld and Dold and of Rosenow⁵ were found, the protocols of the experiments are not given.

At least three different views as to the origin of the toxic substance in anaphylaxis have been advanced. Vaughan and Wheeler⁶ first showed that by the action on protein of alkali in boiling alcohol, substances were formed which, when injected into animals, produced acute toxic symptoms and death. They suggested that anaphylaxis is due to an analogous splitting of the protein, in this case the splitting of the injected protein occurring in the body as a result of the action of ferments or antibodies which have developed as an immunity response to the first injection.

On the other hand, it has been maintained by Nicolle, Weichhardt, Wolff-Eisner and others that the facts may be best explained by assuming that the antibody reaction does not result in a splitting of the protein, but in the case of bacteria, at least, in a lytic action, resulting in the setting free of preformed toxic substances contained within the bodies of the bacteria, endotoxins.

Finally, Wassermann and Keysser⁷ hold that the toxic substance does not arise from the antigen, but is a product of the amboceptor of the serum. They

³ Friedberger, E., and Vallardi, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 94.

⁴ Neufeld, F., and Dold, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 55.

⁵ Rosenow, E. C., *Jour. Infect. Dis.*, 1911, ix, 190.

⁶ Vaughan, V. C., and Wheeler, S. M., *Jour. Infect. Dis.*, 1907, iv, 476.

⁷ Keysser, Fr., and Wassermann, M., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1911, lxviii, 535.

think that the so called anaphylatoxin may be produced by the interaction of inactive substances, as *kieselguhr*, with immune serum, in this case the inactive substance taking the place of the bacterial antigen.

It is impossible here to review all the evidence bearing on this subject, but the experiments to be detailed below will be considered as having a bearing on these various theories.

Neufeld and Dold⁷ showed that in order to produce similar toxic substances from bacteria, the presence of serum is unnecessary, but that by simple extraction of bacteria in salt solution containing 0.1 per cent. of lecithin, such toxic substances could be obtained. Rosenow⁸ then showed that if pneumococci are merely placed in salt solution and allowed to remain in this solution for forty-eight hours at 37° C., the extract so formed is toxic and on injection intravenously into guinea pigs, symptoms and acute death, like that seen in serum anaphylaxis, result. The nature of the change occurring in the bacteria which renders them toxic has been considered by Rosenow to be a true autolysis. He thinks that, owing to the activity of the ferments contained in the bacterial cell, self digestion occurs and the products of this protein digestion are toxic. He thinks an identical or analogous change occurs within the body of the infected host. Upon the property of self digestion, according to him, depends the virulence of the infecting organism. If the organism autolyzes readily, it is therefore virulent; if it autolyzes only slightly, little of the poison is set free and the organism possesses slight virulence.

Since the symptoms produced by the injection of anaphylatoxin or bacterial extracts resemble very remotely those occurring during the pneumococcus infection, it is not certain that this poison has any relationship with the symptoms of the natural infection. Nevertheless, the production of a toxic substance from the bodies of pneumococci is of great importance since, as is well known, the bodies of pneumococci killed by heat are slightly toxic under ordinary circumstances, and large amounts may be injected into susceptible animals, namely, the rabbit and guinea pig, without producing marked effects. This poisonous extract may be of great importance in the pathogenesis of the disease, but in order to establish this, it will first be necessary to learn more concerning the nature of the substance and how it is produced.

Hence experiments were undertaken to confirm or disprove the observations of others as to the main fact, and secondly, by modification of the conditions, to learn how toxic extracts may uniformly be prepared, in order that they may be studied further.

⁷ Neufeld, F., and Dold, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 1069.

⁸ Rosenow, E. C., *Jour. Infect. Dis.*, *loc. cit.*

EXPERIMENTS TO DETERMINE THE TOXICITY FOR GUINEA PIGS OF
EXTRACTS OBTAINED BY EXTRACTION OF PNEUMOCOCCI
IN SALT SOLUTION.

The first set of experiments consisted in determining the toxicity of extracts obtained by keeping our stock organism, designated as N-I, in salt solution for various lengths of time. This organism was obtained from Professor Neufeld and was originally isolated from the sputum of a patient with pneumonia. It is the one which has been largely employed by him in his experiments and in the production of his immune serum. It has been frequently passed through mice and possesses high virulence for these animals, 0.000001 of a cubic centimeter of a bouillon culture uniformly killing one of these animals in less than twenty-four hours. It readily retains its virulence on artificial culture medium, and for this reason it is especially suitable for experimental work and has therefore been largely employed in our laboratory. It is a typical pneumococcus, autolyzes readily in salt solution and is easily soluble in bile. The cultures used in this set of experiments were, in most instances, the first or second passages from mice, though in some cases they had been on artificial medium for as many as six transfers.

In all these tests the organisms were grown in bouillon and were removed from the medium by centrifugalization in order to make sure that we were dealing only with the bodies of the bacteria and not with substances derived from the culture medium. If solid medium is employed, it is almost impossible to obtain the bacteria free from small particles of the medium which may not only furnish toxic substances, but, unless the extract be carefully centrifugalized, when injected into the circulation of animals, may cause death by mechanical stoppage of vessels. The objection to the use of solid medium is especially important in the case of blood or serum agar. The presence of small amounts of the complex blood or serum in the mixture renders the analysis of the phenomenon difficult since it cannot be stated with certainty that the effects obtained are due primarily to pneumococci. Wassermann and others have claimed that shock and death like that seen in anaphylaxis may be due to the injection of foreign serum alone.

The culture medium employed in these experiments was a plain bouillon reaction, approximately $+0.6$ to phenolphthalein. In this medium pneumococci grow well, causing a dense uniform clouding of the medium in eighteen to twenty-four hours. At this time the medium still reacts faintly alkaline to litmus. The number of bacteria per cubic centimeter in a twenty-four hour culture of N-I is usually between 250,000,000 and 270,000,000. Since this number was determined several times, the number of bacteria injected is usually designated as those contained in a given number of cubic centimeters of the culture medium. From this a fair idea of the actual number of bacteria injected may then be calculated.

The bacterial bodies having been sedimented by centrifugalization, the supernatant fluid is carefully and completely pipetted away. In the first experiments the salt solution was added directly to this sediment and an emulsion was made by means of a pipette. In all the later experiments, the bacterial bodies were washed in 0.85 per cent. salt solution and again thrown down in the centrifuge and the emulsion made from these washed bacteria. It was found by experiment that moderate amounts of the medium alone may produce considerable effects in the injected animal,—even death, if larger amounts are employed. The symptoms are probably caused by peptone. It is extremely important in experiments of this kind that effects are not ascribed to toxic substances derived from the bacteria, when they are really due to peptone or other substances contained in the medium. After adding the desired amount of salt solution to the bacterial sediment, a uniform emulsion was made by means of a pipette and this emulsion, in a loosely stoppered bottle, was placed in a thermostat or on ice for the desired length of time.

In certain of the experiments the emulsion was covered by a layer of ether, as advised by Rosenow, but in the larger number this was not done. That lysis of the bacteria goes on more rapidly or completely when ether is added was not confirmed. Moreover, what is more important, the injected animals may show marked effects due to the ether alone.

Various concentrations of bacteria in the salt solution used for the extract were employed, the same culture being employed in making extracts of various concentrations. In most of the extracts, 1 c.c. represented the bacteria from 10 to 20 c.c. of bouillon culture, but extracts of lesser and greater concentration were also employed. Usually 6 to 8 c.c. of the extract were injected, but as much as 10 c.c. and as little as 1 c.c. were also inoculated. The extracts injected represented the bacterial bodies obtained from as little as 6 c.c. to as much as 180 c.c. of bouillon culture, or 1.5 billion to 45 billion bacteria. Most tests were made with doses of extract representing about 10 to 20 billion bacteria. After standing in the incubator for twenty-four hours, the fluid usually clears and the bacteria fail to stain entirely by the Gram method, and stain faintly or not at all with the ordinary dyes. Frequently even after autolysis is said to be complete, the forms of the bacteria in the centrifugalized sediment may still be made out when the preparation is stained with the Manson stain. Most extracts were allowed to remain at 37°C . for from twenty-four to forty-eight hours. Extracts were tested that had been kept at 37°C . for as short a time as eighteen hours and as long as six days. Extracts kept on ice from

twenty-four hours to as long as twenty-five days were tested also. Again extracts were used that had been kept at 37° C. for from twenty-four to forty-eight hours and then for various lengths of time on ice. No special effort was made to determine the extent of lysis by a determination of soluble nitrogen, or by determining the changes produced in the rotation of polarized light by the solutions. The tests we have made with the polariscopic method have shown that if changes occur, they are so slight as to be negligible. Certain of the changes recorded by others and on which stress has been laid, have been so slight as to be within the limits of error of the best instruments. The changes undergone by the emulsion are by no means entirely proteolytic, and to employ this method, under the given conditions, as an indication of proteolytic cleavage, is a doubtful expedient.

Guinea pigs were used in making the tests in this set of experiments. At first the guinea pigs employed weighed 250 to 340 gm., but in all the later experiments, the animals were smaller, weighing between 200 and 250 gm. The injections were made into the external jugular vein, and usually 4 to 8 c.c. of fluid were injected, but in a few instances as much as 10 c.c. were injected. Of 144 animals 5 showed immediate symptoms following the injection which could be clearly ascribed to the incomplete removal of ether from the extract. In 90 there were no symptoms at all, or symptoms so slight that it did not seem justifiable to consider them as representing more than the shock due to the operation and injection of the considerable amount of fluid. Following the injection certain animals showed some tremor, the hair becoming ruffled and the animal appearing quiet and sick. In 26 instances the symptoms were described as of moderate severity and could possibly be considered analogous to the mild shock seen in anaphylaxis. The animals frequently coughed, had somewhat labored breathing, and marked tremor. In only 23 animals, however, were symptoms seen which could be definitely considered as resembling those seen in true anaphylactic shock. In most of these the symptoms very closely resembled those seen in true anaphylaxis. The animals coughed, voided urine, had marked dyspnea, and in many cases convulsions.

Seven of these animals died acutely within ten minutes. Where autopsy was performed quickly, the heart was found still beating and the lungs were usually voluminous, though in only one instance did the lungs remain distended, taking the shape of the chest and being firm and dry, as seen in typical anaphylactic death. Small focal hemorrhages were seen in the lungs and in two cases sub-epicardial hemorrhage.

In the seven instances the symptoms and autopsy findings may therefore be said to resemble closely those seen in anaphylaxis, and if occurring in guinea pigs following the second injection of protein, would undoubtedly have been considered as typical anaphylactic shock.

Of the other 16 animals showing marked symptoms, 10 recovered. Of the remaining 6, 1 died in one and a half hours, 1 in three and a half hours, 1 in twenty-four hours, and the others in from eight to twelve hours. In addition

to the 5 animals which died within twelve hours, there were 51 animals which showed no, or only moderately acute, symptoms, but which died within twelve hours. These 56 animals presented fairly characteristic symptoms, and at autopsy showed lesions such as have been described as occurring in late anaphylactic death. Even where no marked acute symptoms arose, they all had some dyspnea and coughing and appeared quite sick within an hour of the injection, the hair being ruffled and marked tremors occurring. Many passed bloody urine, others showed a slight bloody frothy discharge from the nostrils; and after periods varying from one and a half to twelve hours they died, frequently in convulsions. At autopsy the peritoneum contained a very little blood-tinged fluid or large amounts of clotted blood. The bladder was frequently distended with dark bloody urine. Focal hemorrhages existed in the wall of the stomach and intestine. Similar hemorrhages occurred in the intestine, mainly about the cecum and adjacent portions but sometimes throughout. The lungs were moderately distended and usually dry, although sometimes edematous, and focal hemorrhages, from pin-point size up to 2 to 3 mm. in diameter, were scattered throughout almost constantly. Frequently there were well marked epicardial hemorrhages. Cultures and films made from the heart's blood showed in some cases the presence of a moderate number of organisms; in others the cultures were sterile. That the symptoms and death were not due to infection was rendered most probable by the fact that in many cases no organisms could be demonstrated and also because in guinea pigs, no matter how severe the degree of infection, death rarely occurs within twelve hours. Moreover, the pneumococci in this series of experiments were not very virulent for guinea pigs. Undoubtedly in many of the extracts living organisms were present, but the fact that 71 out of the 144 animals recovered, many of them having received large injections of concentrated extracts, shows that the number of living virulent organisms in the extracts was comparatively small. It would seem, then, that these 56 animals died of intoxication induced by substances contained in the extract.

In the report by Dold dealing with the intoxication of guinea pigs by extracts of pneumococci in lecithin containing salt solution, it is noted that most of the animals did not die acutely, but in from one to six hours. Notwithstanding this late death, they are considered to have shown the features of anaphylactic death. Undoubtedly the kind of intoxication and death occurring in the fifty-six animals described in our series corresponds to that described by Dold.

An attempt to bring the symptoms and kind of death into relation with any of the variables in the preparation of the extract or mode of injection has been fruitless. Some of the animals dying acutely were injected with extracts of low concentration, and others with extracts of high concentration. The same is true of the animals dying late. Nor did the period of autolysis seem to have any

effect on the result. One of the animals dying acutely had been injected with extract kept forty-eight hours at 37° C., and four days on ice; another with extract kept forty-eight hours at 37° C., and five days on ice; another with extract kept forty-two hours at 37° C., and four days on ice; two others with extracts kept forty-eight hours at 37° C., and the remaining two with extracts kept for twenty-four hours at 37° C. Frequently, it was impossible to reproduce the phenomena, even though the conditions were exactly identical so far as could be determined. In two of the animals dying acutely, a few drops of blood were allowed to flow into the syringe and mix with the extract in the syringe before injection. It was thought that some reaction might have taken place between the small amount of blood and the extract. This procedure, however, was repeated in eight other guinea pigs without producing acute death in any case. In one instance, one cubic centimeter of fresh blood was added to the extract before injection. Seven guinea pigs were injected with extracts to which had been added various amounts of fresh guinea pig complement, and the mixture had been kept at 37° C. for from one to two hours. Acute death occurred in only one of these animals. This was quite like that occurring in anaphylactic shock and occurred in an animal which had been injected with three cubic centimeters of extract plus four cubic centimeters of complement, and the mixture kept at 37° C. for two hours.

The result of this set of experiments was therefore inconclusive, in so far as finding a means of producing an extract that would uniformly kill guinea pigs with symptoms like those seen in anaphylaxis. Although seven animals died acutely presenting symptoms resembling acute anaphylactic death, and fifty-six died within twelve hours presenting features resembling those seen in the more chronic forms of anaphylactic death, the results were not constant and striking enough to permit a determination of the exact conditions under which extracts producing acute death may be constantly obtained.

It was thought that possibly the failure to obtain more constant and striking results might be due to the race of organism studied, and consequently a series of tests was made with extracts prepared

from two other races. Both the organisms were obtained from pneumonic patients and had high virulence for mice. The extracts were tested on forty guinea pigs, none of which died acutely. Eight showed moderate or severe symptoms following the injection, and eighteen died within twelve hours. At autopsy these showed conditions resembling those previously described as present in the animals dying late.

Extracts were also prepared from cultures of pneumococci made in bouillon directly from the blood of pneumonia patients. Two extracts made from two races of pneumococci (Davis O' and Baron O') were tested, but the results did not differ from those previously mentioned.

Extracts were also prepared from two cultures kindly sent us by Dr. Rosenow. The following is a brief report of the results obtained with them.

Culture 622^a (R-I).—Blood agar. Received Dec. 12, 1911. This organism was "isolated from the blood on the second day of the disease in a case of lobar pneumonia, and has been passed through four guinea pigs."

Three extracts, prepared as in previous experiments, were tested on four guinea pigs. None showed marked symptoms. One died within twelve hours and showed the lesions previously described.

Culture 602ⁿ (R-II).—Blood agar. Received Dec. 12, 1911. This organism was "originally isolated from consolidated lung after death and has been passed through thirty-one guinea pigs."

Two extracts were prepared in the usual way and tested on four guinea pigs. None showed marked symptoms. All recovered.

A test of the virulence of this organism (602³¹) showed that while it had high virulence for mice, its virulence for guinea pigs, in spite of the thirty-one passages, was not very high. An attempt was therefore made to raise its virulence for guinea pigs before retesting the extract. The pneumococci were passed through a series of guinea pigs, inoculating directly from the abdominal cavity of one guinea pig to that of the next. After passage through twelve guinea pigs in this way, the virulence had been somewhat increased.

Cultures were made at various times from the peritoneal cavity of the dead guinea pigs and extracts were made from the cultures. The extracts were made from cultures of the first, sixth, seventh, twelfth, and thirteenth passages, in plain broth, and in 2, 6, and 8 per cent. glucose broth. All were covered with ether during autolysis. The extracts were tested on sixteen guinea pigs, none of

which died acutely, and which showed symptoms like those seen in anaphylaxis. Eight guinea pigs showed practically no early symptoms whatever. Three showed quite marked immediate symptoms of collapse, which seemed to be associated with incomplete removal of the ether. Only two showed marked symptoms which were like those seen in acute anaphylaxis. Six recovered. Eight died within twelve hours showing symptoms and autopsy findings like those seen in the similar group with other extracts. One died in twenty-four hours.

Hence, so far as tested, these extracts did not differ materially in their effect from those prepared from the cultures which were less virulent for guinea pigs.

The virulence for guinea pigs of the N-I culture used in the previous experiments was low. While 0.000001 of a cubic centimeter of the twenty-four hour culture was uniformly fatal for mice within twenty-four hours, as large quantities as the bacteria from five cubic centimeters of culture did not kill guinea pigs uniformly. Of six guinea pigs injected with 1.1 cubic centimeters, four recovered and two only died after eight days.

An effort was made to increase the virulence of this race for guinea pigs in order to determine whether the inconclusive results might be due to the low virulence for these animals. The same method was employed to raise the virulence of this organism as that used in the case of organism R-II. After passing through the abdominal cavities of fourteen guinea pigs, a culture was made in 6 per cent. glucose bouillon and an extract prepared. This was tested in five guinea pigs. One showed marked symptoms and died acutely, in a manner resembling acute anaphylactic death. The others recovered.

RESULTS OF THE STUDY OF SALT SOLUTION EXTRACTS.

As compared with the reports of others, the results of the study of the toxicity of extracts of pneumococci in salt solution have been disappointing. The observations of Dold would not lead one to expect to find the extracts acutely toxic with any great regularity, while those of Rosenow would indicate that acutely toxic extracts are obtained without difficulty. Of the 213 guinea pigs we injected with salt solution extracts of the several races of pneumococci, only eight died acutely. In these the symptoms present and the autopsy

findings resembled those present in anaphylaxis. Eighty-three of the animals died in from one to twelve hours under fairly characteristic conditions, such as are sometimes seen in sensitized animals dying several hours following the second injection of a protein. So far we have not found the exact conditions upon which the development of toxicity in the salt solution extracts depends. It is, of course, possible that the varieties of guinea pigs are responsible for the irregular results. Rosenow⁹ states in his last communication that the susceptibility of guinea pigs to the action of extracts is greatly increased by starvation. We have made no changes in the regular feeding of the animals tested. Further study may show that factors which we have overlooked in the preparation of the medium or mode of injection, are responsible for the failures, but without a knowledge of these factors any quantitative study of the problem will be impossible.

STUDY OF PERITONEAL WASHINGS.

Since the salt extracts of pneumococci did not show as high toxicity as was anticipated, it was held possible that in the peritoneal cavity the solution of the bacteria might go on at a more rapid rate, from which cavity solutions might be obtained of greater and more constant toxicity.

Although the experiments on rabbits, mentioned early in the paper, to determine the toxicity of the blood of infected rabbits proved negative, yet it seemed possible that in the case of peritoneal infection, the toxin might not be so quickly fixed, or might be formed in such overwhelming amount that it might be found later. Friedberger and Nathan¹⁰ have reported finding "anaphylatoxin" in the peritoneal cavities of guinea pigs into which *Bacillus prodigiosus* had been injected. Rosenow also states that the peritoneal exudates following the injection of pneumococci are toxic.

The guinea pigs from which the exudate was obtained were infected by intraperitoneal injection. As soon as possible after death the peritoneal cavity was opened and washed out with a small amount of normal salt solution.

⁹ Rosenow, E. C., *Jour. Infect. Dis.*, 1912, xi, 94.

¹⁰ Friedberger, E., and Nathan, E., *Ztschr. f. Immunitätsforsch., Orig.*, 1911, ix, 444.

Usually 10 to 15 c.c. sufficed to obtain sufficient fluid for the subsequent injection. After mixing thoroughly with the small amount of exudate usually present, the fluid was removed by a pipette and placed in a powerful centrifuge in which the cells and the larger number of bacteria were removed. The clear fluid was injected into the jugular vein of the animals used in the test. Since it was possible that the pneumococcus which had grown in the animal might be more susceptible to lysis or yield toxin more readily than those grown on artificial media, the sediment obtained on centrifugalization of the peritoneal washings, consisting of bacteria and a varying number of cells, had added to it 10 to 15 c.c. of salt solution and the resulting emulsion was placed at 37° C. for twenty-four to forty-eight hours, after which it was again centrifuged and the supernatant fluid injected into the vein of guinea pigs. Peritoneal washings were obtained from nine guinea pigs which had been infected with pneumococcus R-II. In two instances sufficient washings were obtained for testing each on two guinea pigs, so that in all eleven guinea pigs were injected.

Of these eleven animals, eight showed immediate symptoms like those seen in anaphylaxis, two showed but slight symptoms following the injection. One animal died during the injection. In this case the fluid was thick and viscid and death may have been due to mechanical causes. Of the remaining ten animals, four died within a few minutes with typical features of anaphylactic death and with characteristic autopsy findings. The other six all died in less than twelve hours, and at autopsy, findings, previously described in other animals dying subacutely, were found. That infection was not primarily the cause of death in these animals is shown by the fact that cultures and smears from the heart's blood were usually negative. Of course most of them would undoubtedly have died later from infection, but it seems probable that in these instances death was due to toxic substances transferred from the previously infected animals.

Seven animals were injected with the extract obtained by allowing the sediment to undergo autolysis in salt solution, as previously noted. The results were not nearly so striking as those in which the peritoneal washings were immediately employed.

The direct injection of peritoneal washings into the veins of other guinea pigs gave results which were important and striking. That four out of the ten animals had acute shock and death simulating that seen in anaphylaxis indicates that the development of the toxic substance must be much more constant in the abdominal cavity of the guinea pig than in the test-tube, if we assume that the same

process is active in the two cases. However, in the animal body the conditions are much more complex. In order to obtain an active extract under as simple conditions as possible, further efforts were directed to prepare it outside the body.

STUDY OF CHLOROFORM EXTRACTS.

The toxic effect of pneumococcus is not necessarily due to digestion products of the bacteria, as has been considered to be the case, but may be caused by preformed substances set free on the disintegration of the bodies of the bacteria, the essential process being, therefore, a form of plasmolysis, rather than of autolysis. Various methods were employed in an endeavor to throw the bacterial bodies into solution rapidly under conditions in which autolysis could, with reasonable certainty, be excluded.

Of the procedures, the only one giving any degree of success consisted in the use of chloroform, by which method we were able to obtain fairly constant solutions of the bacterial bodies. The method, however, is troublesome. The mixtures of chloroform and emulsion do not always become clear, and the injection of the solution into the veins of animals did not give constant results.

STUDY OF BILE EXTRACTS.

We next studied the solution of pneumococci obtained by means of bile. It was first shown by Neufeld¹¹ that pneumococci, when treated by bile or solutions of bile salts, readily undergo solution, and the emulsion, which was previously cloudy and opaque, becomes clear and translucent and on staining, the form of the bacterial bodies can no longer be made out. Although Rosenow states that “. . . complete lysis of pneumococci in weak solutions of bile salts (0.5 per cent.) . . . interferes with the production of anaphylatoxic substance . . .,” preliminary experiments performed by us showed that solutions of pneumococci in bile salts, when injected intravenously, were toxic for guinea pigs. In making the solutions, a 2 per cent. solution of sodium cholate in normal salt solution was employed. It was found, however, that with this low dilution of the

¹¹ Neufeld, F., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1900, xxxiv, 454.

bile salt, clearing of various solutions did not always occur with equal rapidity, some solutions clearing more rapidly and others not at all.

An experiment was therefore undertaken to determine the effect of dilutions of sodium cholate on the lysis, the number of bacteria and the amount of sodium cholate being kept constant.

Two sets of tubes were prepared, one set kept at 37° C., the other on ice. It was found that the action of the cholate depends on the concentration of the sodium cholate in the emulsion, rather than on the relation of the amount of the salt to the number of bacteria present. 0.1 c.c. of a 2 per cent. sodium cholate solution was found to be much more efficient in producing solution of the given number of bacteria, when they were in a 1 c.c. emulsion, than even 0.2 c.c. when the bacteria were made into a 10 c.c. emulsion. It was found also that while lysis occurs somewhat better at 37° C., yet complete solution, when the proper mixtures are employed, may be obtained when the entire process is carried out on ice.

Even bearing in mind the above fact, however, solution of pneumococci could not always be readily obtained, even when the proper mixtures were employed. It was found that with bacteria grown in glucose bouillon, even when washed in salt solution, solution did not readily occur. It was found that when pneumococci were grown in plain bouillon and centrifugalized, then washed with the supernatant fluid from the glucose bouillon culture, the organisms, which otherwise readily dissolved, were now insoluble. It was therefore evident that the insolubility was due to something in the glucose bouillon fluid which adhered to or was absorbed by the bacteria. It seemed probable that this was acid, and this was found to be the case. Very small amounts of acid in the emulsion prevent solution of the bacteria, probably owing to the fact that cholic acid, which is insoluble, is set free.

A test was therefore made to determine the optimum reaction for lysis with bile. As is well known, by mixing primary and secondary sodium phosphate, solutions may be obtained which retain to a considerable degree their original reaction, in spite of changes occurring in the medium which would otherwise influence the reaction. By adding mixtures of primary and secondary phosphates in various proportions to the tubes containing bacteria and bile, it was found that lysis goes on most rapidly when mixtures of one

part primary to seven to eight parts secondary phosphate are employed. This gives about the reaction of blood serum.

EXPERIMENTS WITH GUINEA PIGS.

Sixty-three guinea pigs, weighing 200 to 250 grams each were injected with various amounts of the cholate extracts of pneumococci. Forty-nine showed marked symptoms similar to those seen in acute anaphylaxis, one showed moderately severe symptoms, and thirteen showed only slight symptoms or none at all. Of the sixty-three animals, thirty-seven died acutely under conditions resembling those seen in acute anaphylaxis, fifteen died in from one half to twelve hours and showed hemorrhages and other features previously described as occurring in animals dying after a few hours, one animal died during the injection,—the cause of death could not be determined,—while ten recovered. It will thus be seen that a large proportion of the animals died acutely in the manner seen in acute anaphylaxis, far more deaths occurring than with extracts prepared by extraction in salt solution. It must be remembered that these results were obtained by employing extracts prepared in various ways. The results would be much more striking if they included only animals injected with extracts prepared in the manner which we now know is most efficient for producing a toxic effect.

In the above series, the extracts injected contained only small amounts of sodium cholate. One animal dying acutely received but 0.04 of a cubic centimeter of the cholate solution. All but seven of the animals received less than 0.2 of a cubic centimeter of the 2 per cent. solution. Six received between 0.2 and 0.3 of a cubic centimeter, but of these only three died acutely. One received more than this, but it died only after four and a half hours. That the symptoms and death are not dependent upon the sodium cholate was shown, moreover, by control tests in which animals were injected with different amounts of the sodium cholate solution. One received 0.2, one 0.4, and one 0.6 of a cubic centimeter of the cholate solution in eight cubic centimeters of sodium chlorid solution. None of them showed symptoms and none died. It is quite probable that very much larger amounts could be injected without producing symptoms. It was thought possible that the reaction

might be due to some non-specific combination between the protein and the sodium cholate, of the nature of a physical reaction. But a guinea pig injected with a solution of egg albumin treated with sodium cholate solution showed no symptoms.

The extracts injected contained the substance of varying amounts of bacteria. The race of pneumococcus described as N-I was exclusively employed. After centrifugalizing from the bouillon culture, the pneumococci were washed in salt solution. Some of the extracts were prepared from quite large numbers of bacteria,—as many as those obtained from centrifugalization of 160 c.c. of the culture. The larger number, however, were prepared from the bacteria obtained by centrifugalization of 60 to 80 c.c. of the culture. Positive results were obtained also from smaller amounts. In one instance, acute symptoms and death developed after the injection of an extract prepared from the bacteria obtained from only 25 c.c. of the culture.

The solution of the bacteria was obtained by leaving the mixture on ice or at 37° C. for various lengths of time, usually from one half to several hours. In three instances acute symptoms and death were seen after the injection of extracts that had been prepared in the cold and kept on ice for only thirty minutes before injection. Three positive results were obtained with extracts that had been kept at 37° C. only ten minutes, and in five cases the mixture had been kept only fifteen minutes at 37° C. It is thus seen that the reaction which occurs between the bile salt and the bacteria occurs very rapidly, only sufficient time being required to bring the bacterial bodies into solution. Since by experiment we have learned how to produce this result most rapidly, it has been found that positive results may be obtained almost invariably, by the injection of 6 c.c. of an extract prepared from the bacteria in 50 to 60 c.c. of culture, the mixture having been kept half an hour at 37° C. The best method we have found is the following: 1,000 c.c. of a twenty-four hour culture are centrifugalized, the bacteria are washed once in salt solution, and after the second centrifugalization, the supernatant fluid is removed as completely as possible. Five c.c. of salt solution are now added, and 5 c.c. of a mixture of primary and secondary sodium phosphate solutions in the proportion of 1 to 8; a thick emulsion is thus produced, after which 2 c.c. of the 2 per cent. sodium cholate solution are added and mixed, and the mixture is placed in the water bath at 37° C. for half an hour. Sufficient salt solution is then added to bring the total up to 100 c.c., and after thorough mixing, the extract is ready for injection. The extract need not be centrifugalized unless it contains foreign particles. Six c.c. of such an extract contains the bacterial substance from the bacteria contained in 60 c.c. of the bouillon culture and 0.12 c.c. of the sodium cholate solution.

The symptoms produced and the results obtained are proportionate to the amount of extract injected, though there appear to be some individual differences in sensitiveness in the different guinea pigs. Yet two extracts prepared exactly alike do not necessarily have identical toxicity, a fact depending in part upon the varying

number of bacteria contained in different cultures. Each extract, therefore, has to be independently standardized.

TESTS OF THE BILE EXTRACTS IN RABBITS.

The toxicity of the bile extracts of pneumococci has been further studied by injection into rabbits. Rabbits weighing 700 to 1,200 grams were employed, and the injections were made into the ear veins. A considerable number of the rabbits injected with fifteen to twenty cubic centimeters of the extract died acutely in from two or three to thirty minutes. Of twenty-nine rabbits injected, eleven died acutely, eleven died in from one to twelve hours, and seven recovered or died of infection in from one to three days.

A rabbit dying within a few minutes showed, usually, the following symptoms: After the injection it would be quiet for a few minutes, then would suddenly start to run violently across the floor during which process it usually fell, and frequently would throw itself about on the floor. While on its side it would continue to make running motions with its legs, its head would be drawn back, its eyes prominent, the pupils usually dilated, the animal would cry out, make a few convulsive movements, and then died. When the symptoms were a little less acute, the progression of the disturbance could be better followed. The following protocol describes fairly well what occurred in a typical experiment:

Rabbit 5 E.—Weight 700 gm.

April 5. Injected into the ear vein 15 c.c. of an extract made by treating the bacteria from 200 c.c. of a bouillon culture with 0.7 c.c. of a 2 per cent. solution of sodium cholate. The mixture was kept on ice for thirty minutes before injection. Injection was made at 10:56 A.M. At 10:58 the animal appeared drowsy and showed some muscular weakness. 10:59. Respirations are slower; animal appears sick. 11:01. Marked weakness in legs; animal no longer able to hop about. 11:10. Animal lying on side, not attempting to get up. 11:16. Respirations are shallow, 18 to the quarter, slower than previously. 11:19. Makes jumping motions while lying on side. Heart no longer felt. Respirations shallow. 11:21. Active peristalsis. 11:22. Several gasping respirations. Heart cannot be felt. Pupils dilated. Twitching of muscles noted. 11:23. Animal dead.

Autopsy.—Mesenteric veins markedly dilated. No hemorrhages in wall of intestine or stomach. Stomach firmly contracted. Blood removed from mesenteric vein begins to clot in half an hour; not completely clotted in forty-five minutes. Lungs not especially voluminous; show small areas of emphysema.

Heart: On opening the chest, the left ventricle is seen to be making slow ineffectual beats, the auricles are beating less frequently than the ventricles, then in dissociation. There are no pulsations in the right ventricle unless it be at the tip. The right ventricle is much distended, the muscle opaque. On opening the right ventricle, the wall contracts and becomes crinkled. On scraping, the muscle seems distinctly tougher than usual.

Other animals showed features differing slightly in details from those described. Some of them showed paralysis, beginning in the hind legs, the animals dragging themselves about. In general, muscular weakness was an early condition and death seemed to be due to cardiac, rather than to respiratory involvement. The animals showed in general the features described by Auer as occurring in acute anaphylactic shock in rabbits. A typical animal, seen by Auer, was said by him to behave and to show autopsy findings like those seen in his rabbits.

The animals dying after several hours showed at autopsy changes resembling more those seen in guinea pigs dying late. Focal hemorrhages were an almost constant finding. These were most marked, as in the guinea pigs, in the cecum and adjacent portions of the intestine, in the stomach, and in the lungs. Frequently there was a bloody exudate into the peritoneum and the bladder was filled with bloody urine.

In this series of rabbits, as in the guinea pigs, extracts of various concentration were employed and were prepared by leaving the mixtures at 37° C. or on ice for short periods of time. Typical acute death was obtained with extracts that had been kept at 37° C. for only ten minutes and also from extracts that had been prepared in the cold and kept on ice no longer than thirty minutes. It was finally found that by the injection of fifteen to twenty cubic centimeters of the extract prepared as described on page 660, acute death could almost always be produced. The tolerance of rabbits for sodium cholate was as great as four cubic centimeters of the solution in sixteen cubic centimeters of salt solution without any symptoms whatever. This is a very much larger amount than was injected in any of the experiments, and shows that the effects cannot be due to that substance.

That the result described bears a quantitative relationship to the amount of bacteria injected is shown by the following experiment:

Five rabbits were injected with various quantities of an extract prepared as previously described. One hundred c.c. of extract were prepared from the bacteria in 1 liter of culture medium. One and a half c.c. of sodium cholate solution were used in causing solution which occurred in fifteen minutes at 37° C.

Rabbit No.	Amount of extract injected.	Symptoms.	Result.
1	22 c.c.	Marked	Died, 3 min.
2	20 c.c.	Slight	Died, 4 hrs.
3	15 c.c.	Slight	Died, 11 hrs.
4	10 c.c.	Slight	Died, 12 hrs.
5	5 c.c.	None	Recovered.

Further studies dealing with the properties of the bile extract will be reported later. It may be stated, however, that tests of the effect of heating the extract gave the following results:

Guinea pig No.	Extract heated.	Result of injection.	
		Symptoms.	Final results.
1	60-65° C. for 1 hr.	None	Recovery.
2	55° C. for ½ hr.	None	Recovery.
3	55° C. for ½ hr.	Very slight	Recovery.
4	55° C. for ½ hr.	Very slight	Recovery.
5	45° C. for 1 hr.	Marked	Died, 30 min.

Controls with the extracts employed unheated showed marked symptoms and acute death.

Rosenow states that the toxic substance in salt solution extracts is destroyed by heating for one half hour at 60° C., but Dold¹² that the toxic extracts are quite resistant to heat; they are weakened by two hours heating to 56° to 58° C., but are not destroyed. The results of our tests of a number of bile extracts seem to show conclusively that they are thermolabile. Possibly the reason that the bodies of bacteria killed by heat are not toxic is because the thermolabile toxic substance is destroyed.

The study of the extract of pneumococcus in bile solution has shown that its toxicity for rabbits and guinea pigs is greater and more constant than is the extract in salt solution. Further work will be required to show its relation to the poison responsible for the symptoms of pneumococcus infection in animals and of lobar pneumonia in man. The fact that poisons having a similar action are found in the peritoneal exudate of infected animals suggests that these poisons may play a part in pneumococcus infection.

¹² Dold, H., *Das Bakterien-Anaphylatoxin*, Jena, 1912.

These observations also have a bearing on the present theories of anaphylaxis. The fact that the effects produced by this poison, both in guinea pigs and rabbits, are similar to those produced by the second injection of protein, suggests that the poisons concerned are of a similar nature, though this is not necessarily the case. In the present instance there is evidence that the poison does not arise as a result of protein digestion, since it may be set free within half an hour at a temperature of about 4° C. Proteolytic cleavage, due to ferments with which we are familiar, does not occur under these conditions. It is more likely that the toxic substances in the bile extract exist preformed in the bodies of the bacteria and are set free on their dissolution.

CONCLUSIONS.

1. The filtered blood serum of rabbits infected with pneumococci is not toxic.
2. Extracts of pneumococci prepared by keeping emulsions of the bacteria in salt solution at 37° C. for varying periods of time may be toxic, and when injected intravenously into guinea pigs, may produce a train of symptoms followed by acute death resembling that seen in acute anaphylaxis. Such extracts, however, are not uniformly toxic and it has been impossible to discover the exact conditions under which such extracts become toxic.
3. When the centrifugalized peritoneal washings of guinea pigs infected with pneumococci are injected into the circulation of normal guinea pigs, these animals very frequently exhibit symptoms like those seen in acute anaphylaxis, and a considerable proportion of the animals die acutely.
4. When pneumococci are dissolved in dilute solutions of bile salts and the solution resulting is injected intravenously into rabbits and guinea pigs, these animals show with great constancy the same symptoms that are seen in acute anaphylaxis. The solution of pneumococci in bile may occur in ten minutes at 37° C. or in half an hour on ice. This is considered evidence that the toxicity of the solution does not result from digestion of the bacterial protein, but is due to substances preformed in the bacterial cells and set free on their solution. The toxicity of the solution is diminished or destroyed by heating to 55° C. or over.

THE PRESENCE OF PROTECTIVE SUBSTANCES IN HUMAN SERUM DURING LOBAR PNEUMONIA.*

By A. R. DOCHEZ, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

As long ago as 1891, G. and F. Klemperer¹ showed that the blood of patients who had recovered from an attack of pneumonia might protect rabbits against infection with pneumococcus. Since then Neufeld and Haendel² have also demonstrated that such sera may have protective power, not only for rabbits but for mice as well. Other observers have found that this property of post-critical blood serum is not constant, and Strouse,³ and Seligmann and Klopstock,⁴ have even failed to demonstrate it in any instance. The observations previously mentioned, however, seem to indicate that such protective substances may be present at the time of the crisis or afterward.

Considerable speculation has been indulged in, and no little work carried on in order to bring the development of this protective power into direct causal relationship with the crisis in this disease. The crisis is such a clear-cut, striking phenomenon that it would seem that it may depend on some single cause or relatively simple biological reaction, such as the destruction of the bacteria by immune substances or neutralization of toxin by antitoxin. If the blood of animals recovering from the disease constantly contains protective substances, it would be reasonable to assume that the crisis and recovery occur because of the development of such substances.

Observations have also been made to determine the variety of the

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¹ G. and F. Klemperer, *Berl. klin. Wchnschr.*, 1891, xxviii, 833, 869.

² Neufeld, F., and Haendel, *Arb. a. d. k. Gsndhtsamte*, 1910, xxxiv, 166.

³ Strouse, S., *Jour. Exper. Med.*, 1911, xiv, 109.

⁴ Seligmann, E., and Klopstock, F., *Ztschr. f. Immunitätsforsch., Orig.*, 1909-10, iv, 103.

immune bodies developing in the blood, to which the protective power is due. G. and F. Klemperer considered the protective power dependent upon an antitoxic action of the serum. Römer⁵ holds the opinion that the chief action is bactericidal; while recently much stress has been laid on the development of opsonins or bacteriotropins in the serum. In the absence of the demonstration of a pneumococcus toxin, the view that the protective power may be due to an antitoxin is purely hypothetical. Even Römer has not been able to demonstrate the bactericidal power *in vitro* of post-pneumonia serum. Virulent pneumococci are so resistant to the action of opsonins, as detected by the ordinary technique, that even artificial immune sera, having far greater protective power than any post-critical sera so far studied, show very slight if any increase in opsonic power for such virulent organisms. By the use of a different technique, Neufeld and Haendel have demonstrated that such post-critical sera may show an increase of their property of favoring phagocytosis, both *in vivo* and *in vitro*. This property, they consider, depends on the presence of bacteriotropins.

In the effort to find an explanation of the crisis, however, it is of great importance to determine definitely whether protective substances appear constantly in the post-critical serum, and whether the time of their appearance bears any relationship to the time of crisis or recovery. The mere demonstration of the occasional presence of protective substances in post-critical sera is not sufficient to permit drawing any conclusions as to their relationship to the phenomenon.

The blood obtained from a series of patients has therefore been studied to determine whether such substances are present, and if so, to determine the time of their appearance. No effort has been made to determine the kind of immune bodies present.

EXPERIMENTAL PART.

Methods.—The sera for the experiments presented in this paper were obtained by venous puncture of patients with typical lobar pneumonia. The blood was allowed to clot, the serum separated,

⁵Römer, P. H., *Experimentelle und klinische Grundlagen für die Serumtherapie der Pneumokokkeninfektion der menschlichen Cornea (Ulcus Serpens)*, Wiesbaden, 1909.

and kept in the dark on ice until used. The organism employed in each experiment was obtained, when possible, either from the blood or sputum of the patient whose serum was to be tested. The organism in each instance was isolated before the occurrence of the crisis. It has been found of some service, in procuring pure cultures from sputum, to inject at the same time a small quantity of human serum which seems to inhibit the growth of contaminating bacteria. In case the pneumococcus, when isolated, was of low pathogenicity, the virulence was raised by successive animal passages until a dose of 0.000001 of a cubic centimeter of a broth culture was sufficient to kill. White mice of as nearly the same age and size as possible were used as experimental animals. Twenty-four hour broth cultures fresh from animals were used for infection, and the serum and culture to be tested were mixed in varying quantities in the barrel of a syringe and immediately injected intraperitoneally. The animals were watched for from five to ten days, according to the rate at which the culture killed, and those surviving this time were considered protected. A small number of sera were tested for protective power against a typical laboratory strain of pneumococcus; in all other instances, the sera were tested against homologous strains isolated from the patients.

PROTECTIVE POWER OF HUMAN SERA AGAINST A TYPICAL PNEUMOCOCCUS.

Experiment 1.—Serum 123 was obtained from a patient with typical pneumonia of the right lower lobe. The temperature fell by lysis on the seventh day of the disease. The temperature again rose the next day and remained slightly elevated for about six days. The blood culture was negative. The organism used for infection was pneumococcus Neufeld I, a strain of constant high virulence for animals.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, chronic endocarditis.	Serum 123, Mar. 23, 1911, 12 hours before crisis	Serum 123, Mar. 24, 1911, 4 hours after temperature reached normal.	Serum 123, Mar. 27, 1911, 3 days after temperature reached normal. Patient again febrile.	Serum 123, Mar. 30, 1911, 6 days after temperature first reached normal. Patient still febrile.	Serum 123, Apr. 3, 1911, 10 days after temperature first reached normal. Patient again afebrile.	Serum 123, Apr. 13, 1911, 20 days after temperature first reached normal. Patient mal. Patient afebrile.
0.1	0.2	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	—	D. 21 hrs.	D. 21 hrs.
0.01	0.2	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 26 hrs.	D. 21 hrs.	—	D. 21 hrs.	D. 21 hrs.
0.001	0.2	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	—	D. 21 hrs.	D. 26 hrs.
0.0001	0.2	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	Survived.	D. 21 hrs.	D. 21 hrs.	D. 24 hrs.	Survived.
0.00001	0.2	D. 36 hrs.	D. 26 hrs.	Survived.	D. 56 hrs.	D. 36 hrs.	D. 36 hrs.	D. 26 hrs.	D. 36 hrs.
0.000001	0.2	D. 36 hrs.	D. 36 hrs.	Survived.	D. 36 hrs.	D. 36 hrs.	D. 26 hrs.	D. 23 hrs.	Survived.

* D. = died.

Experiment 2.—The serum (405) for this test was obtained from a patient with partial pneumonic consolidation of the left lower lobe. The temperature fell by lysis from the eighth to the ninth day of the disease and remained normal. The blood culture was negative. The protective power of the serum was tested against pneumococcus Neufeld I.

Quantity of culture	Quantity of serum in c.c.	Virulence, no serum.	Control serum, syphilitic.	Serum 405, Dec. 24, 1911, 10 days after temperature reached normal.	Serum 405, Dec. 24, 1911, 6 days after temperature reached normal.	Serum 405, Dec. 24, 1911, 6 days after temperature reached normal.	Serum 405, Jan. 2, 1912, 18 days after temperature reached normal.
1.2	0.2	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 24 hrs.	D. 18 hrs.
1.21	0.2	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 24 hrs.	D. 18 hrs.
1.301	0.2	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 24 hrs.	D. 28 hrs.
0.0001	0.2	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 28 hrs.	D. 28 hrs.
0.00001	0.2	D. 36 hrs.	D. 24 hrs.	D. 18 hrs.	D. 18 hrs.	D. 48 hrs.	D. 48 hrs.
0.000001	0.2	D. 24 hrs.	D. 24 hrs.	D. 18 hrs.	D. 18 hrs.	D. 48 hrs.	D. 48 hrs.

Experiment 3.—The patient whose serum 418 was used in this experiment had a short pneumonia, terminating by lysis from the fourth to the sixth day of the disease. The blood culture was negative. Protection for animals was tested against the stock pneumococcus Neufeld I.

Quantity of culture	Quantity of serum in c.c.	Virulence, no serum.	Control serum, syphilitic.	Serum 418, Dec. 24, 1911, 10 days after first normal temperature.	Serum 418, Jan. 8, 1912, 10 days after first normal temperature.
1.2	—	—	—	—	D. 18 hrs.
1.21	—	—	—	—	D. 28 hrs.
1.301	—	—	—	—	D. 18 hrs.
0.0001	—	—	—	D. 28 hrs.	D. 28 hrs.
0.00001	—	—	—	D. 36 hrs.	D. 28 hrs.
0.000001	—	—	—	D. 36 hrs.	D. 36 hrs.

PROTECTIVE POWER OF HUMAN SERA AGAINST HOMOLOGOUS PNEUMOCOCCI

Experiment 4.—The serum 419 for this experiment was obtained from a patient with partial pneumonic consolidation by lysis on the tenth day, although the temperature remained slightly elevated for six days thereafter. The pneumococcus used for protection was obtained from the patient's blood on the seventh day of the disease. When first injected, 0.5 c.c. of a twenty-four hour culture died in 48 hours. The virulence was raised by successive passages until animals died in forty-eight hours.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence: no serum.	Control serum, syphilis.	Serum 119, Mar. 20, 1911, 3 days before beginning of temperature fall.	Serum 119, Mar. 21, 1911, 2 days before beginning of temperature fall.	Serum 119, Mar. 22, 1911, 1 day before beginning of temperature fall.	Serum 119, Mar. 23, 1911, 3 hours after critical drop in temperature.	Serum 119, Mar. 25, 1911, 2 days after critical drop in temperature. Patient still febrile.	Serum 119, Mar. 27, 1911, 4 days after critical drop in temperature. Patient still febrile.	Serum 119, Mar. 28, 1911, 5 days after critical drop in temperature. Patient still febrile.	Serum 119, Mar. 30, 1911, 7 days after critical drop in temperature. Patient afebrile.	Serum 119, Mar. 31, 1911, 8 days after critical drop in temperature. Patient afebrile.	Serum 119, Apr. 10, 1911, 18 days after critical drop in temperature. Patient afebrile.
0.1	0.2	—	—	—	D. 24 hrs.	D. 24 hrs.	D. 32 hrs.	D. 29 hrs.	D. 42 hrs.	D. 42 hrs.	D. 32 hrs.	D. 24 hrs.	—
0.01	0.2	—	—	—	D. 42 hrs.	D. 20 hrs.	D. 75 hrs.	D. 42 hrs.	D. 42 hrs.	D. 27 hrs.	—	D. 32 hrs.	—
0.001	0.2	D. 27 hrs.	D. 42 hrs.	D. 42 hrs.	D. 29 hrs.	D. 42 hrs.	D. 66 hrs.	Survived.	Survived.	Survived.	D. 42 hrs.	D. 42 hrs.	—
0.0001	0.2	D. 42 hrs.	D. 42 hrs.	D. 66 hrs.	D. 42 hrs.	D. 66 hrs.	Survived.	Survived.	D. 49 hrs.	D. 42 hrs.	D. 44 hrs.	D. 42 hrs.	D. 45 hrs.
0.00001	0.2	D. 50 hrs.	D. 42 hrs.	D. 32 hrs.	D. 42 hrs.	D. 42 hrs.	Survived.	Survived.	Survived.	D. 42 hrs.	D. 66 hrs.	D. 42 hrs.	D. 66 hrs.
0.000001	0.2	D. 45 hrs.	D. 43 hrs.	D. 42 hrs.	D. 47 hrs.	D. 66 hrs.	Survived.	Survived.	Survived.	Survived.	D. 45 hrs.	D. 96 hrs.	Survived.

Experiment 2.—The serum (405) for this test was obtained from a patient with partial pneumonic consolidation of the left lower lobe. The temperature fell by lysis from the eighth to the ninth day of the disease and remained normal. The blood culture was negative. The protective power of the serum was tested against pneumococcus Neufeld I.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, syphilis.	Serum 405, Dec. 14, 1911, immediately before beginning of lysis.	Serum 405, Dec. 15, 1911, 6 hours after temperature reached normal.	Serum 405, Dec. 21, 1911, 6 days after temperature reached normal.	Serum 405, Jan. 2, 1912, 18 days after temperature reached normal.
0.1	0.2	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	—	—
0.01	0.2	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.	D. 24 hrs.	D. 18 hrs.
0.001	0.2	D. 18 hrs.	D. 18 hrs.	D. 24 hrs.	D. 18 hrs.	D. 24 hrs.	D. 18 hrs.
0.0001	0.2	D. 18 hrs.	D. 24 hrs.	D. 24 hrs.	D. 36 hrs.	D. 24 hrs.	D. 28 hrs.
0.00001	0.2	D. 36 hrs.	D. 48 hrs.	D. 28 hrs.	D. 36 hrs.	D. 28 hrs.	D. 28 hrs.
0.000001	0.2	D. 28 hrs.	D. 28 hrs.	D. 36 hrs.	D. 48 hrs.	D. 48 hrs.	D. 48 hrs.

Experiment 3.—The patient whose serum (418) was used in this experiment had a short pneumonia, terminating by lysis from the fourth to the sixth day of the disease. The blood culture was negative. Protection for animals was tested against the stock laboratory pneumococcus Neufeld I.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, syphilis.	Serum 418, Dec. 24, 1911, 2 days before beginning of lysis.	Serum 418, Dec. 30, 1911, 1st day of normal temperature.	Serum 418, Jan. 8, 1912, 10 days after first normal temperature.
0.1	0.2	—	—	—	—	D. 18 hrs.
0.01	0.2	—	—	D. 18 hrs.	—	D. 28 hrs.
0.001	0.2	—	—	D. 18 hrs.	—	D. 18 hrs.
0.0001	0.2	D. 18 hrs.	D. 24 hrs.	D. 28 hrs.	D. 28 hrs.	D. 28 hrs.
0.00001	0.2	D. 36 hrs.	D. 48 hrs.	D. 36 hrs.	D. 36 hrs.	D. 28 hrs.
0.000001	0.2	D. 28 hrs.	D. 28 hrs.	D. 28 hrs.	D. 36 hrs.	D. 36 hrs.

PROTECTIVE POWER OF HUMAN SERA AGAINST HOMOLOGOUS PNEUMOCOCCI.

Experiment 4.—The serum (119) for this experiment was obtained from a patient with typical lobar pneumonia, terminating by lysis on the tenth day, although the temperature remained slightly elevated for six days thereafter. The pneumococcus used for infection was obtained from the patient's blood on the seventh day of the disease. When first isolated, 0.5 c.c. of a twenty-four hour broth culture failed to kill a mouse. The virulence was raised by successive animal passages until 0.000001 c.c. was fatal for mice in forty-eight hours.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence: no serum.	Control serum, syphilis.	Serum 119, Mar. 20, 1911, 3 days before beginning of temperature fall.	Serum 119, Mar. 21, 1911, 2 days before beginning of temperature fall.	Serum 119, Mar. 22, 1911, 1 day before beginning of temperature fall.	Serum 119, Mar. 23, 1911, 3 hours after critical drop in temperature.	Serum 119, Mar. 25, 1911, 2 days after critical drop in temperature. Patient still febrile.	Serum 119, Mar. 27, 1911, 4 days after critical drop in temperature. Patient still febrile.	Serum 119, Mar. 28, 1911, 5 days after critical drop in temperature. Patient still febrile.	Serum 119, Mar. 30, 1911, 7 days after critical drop in temperature. Patient afebrile.	Serum 119, Mar. 31, 1911, 8 days after critical drop in temperature. Patient afebrile.	Serum 119, Apr. 10, 1911, 18 days after critical drop in temperature. Patient afebrile.
0.1	0.2	—	—	—	D. 24 hrs.	D. 24 hrs.	D. 32 hrs.	D. 29 hrs.	D. 42 hrs.	D. 42 hrs.	D. 32 hrs.	D. 24 hrs.	—
0.01	0.2	—	—	—	D. 42 hrs.	D. 20 hrs.	D. 75 hrs.	D. 42 hrs.	D. 42 hrs.	D. 27 hrs.	—	D. 32 hrs.	—
0.001	0.2	D. 27 hrs.	D. 42 hrs.	D. 42 hrs.	D. 29 hrs.	D. 42 hrs.	D. 66 hrs.	Survived.	Survived.	Survived.	D. 42 hrs.	D. 42 hrs.	—
0.0001	0.2	D. 42 hrs.	D. 42 hrs.	D. 42 hrs.	D. 42 hrs.	D. 66 hrs.	Survived.	Survived.	D. 49 hrs.	D. 42 hrs.	D. 44 hrs.	D. 42 hrs.	D. 45 hrs.
0.00001	0.2	D. 50 hrs.	D. 42 hrs.	D. 42 hrs.	D. 42 hrs.	D. 42 hrs.	Survived.	Survived.	Survived.	D. 42 hrs.	D. 66 hrs.	D. 45 hrs.	D. 66 hrs.
0.000001	0.2	D. 45 hrs.	D. 43 hrs.	D. 42 hrs.	D. 47 hrs.	D. 66 hrs.	Survived.	Survived.	Survived.	Survived.	D. 45 hrs.	D. 96 hrs.	Survived.

Experiment 5.—The serum (132) was obtained from a patient with lobar pneumonia, terminating by lysis on from the third to the sixth day of the disease. The pneumococcus against which the specimens of sera were tested was isolated from the sputum, and the virulence raised by animal passage until 0.0001 c.c. was fatal for mice in forty-eight hours. The blood culture was negative.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, endocarditis.	Serum 132, Apr. 17, 1911, 1 day before drop in temperature.	Serum 132, Apr. 18, 1911, during falling temperature.	Serum 132, Apr. 20, 1911, 2 days after drop in temperature. Patient still febrile.	Serum 132, Apr. 23, 1911, 5 days after drop in temperature. Patient afebrile.
0.1	0.2	D. 30 hrs.	D. 30 hrs.	D. 41 hrs.	D. 30 hrs.	D. 41 hrs.	D. 41 hrs.
0.01	0.2	D. 41 hrs.	D. 41 hrs.	D. 30 hrs.	Survived.	Survived.	Survived.
0.001	0.2	D. 41 hrs.	Survived.	Survived.	Survived.	Survived.	Survived.
0.0001	0.2	D. 41 hrs.	Survived.	Survived.	Survived.	Survived.	Survived.
0.00001	0.2	Survived.	Survived.	Survived.	Survived.	Survived.	Survived.
0.000001	0.2	Survived.	Survived.	Survived.	Survived.	Survived.	Survived.

Experiment 6.—The patient from whom the serum (145) used in this experiment was obtained had typical lobar pneumonia. The temperature fell by lysis on the ninth day of the disease, rose again the next day, and remained slightly elevated until the fifteenth day, when it again returned to normal. The patient died thirty days later from a post-pneumonic nephritis. The organism was obtained from the sputum and was the so called *Pneumococcus mucosus*. It was of extremely high virulence for mice. The blood culture was negative.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence ; no serum.	Control serum, nephritis.	Serum 145, Apr. 5, 1911, 6 days before first normal temperature.	Serum 145, Apr. 8, 1911, 3 days before first normal temperature.	Serum 145, Apr. 11, 1911, day that temperature first reached normal	Serum 145, Apr. 15, 1911, 4 days after first normal temperature : still slight fever.	Serum 145, Apr. 20, 1911, 9 days after first normal temperature. Patient afebrile.
0.1	0.2	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.
0.01	0.2	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.
0.001	0.2	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 21 hrs.	D. 45 hrs.	D. 21 hrs.	D. 21 hrs.
0.0001	0.2	D. 21 hrs.	D. 29 hrs.	D. 21 hrs.	D. 25 hrs.	Survived.	D. 21 hrs.	D. 25 hrs.
0.00001	0.2	D. 21 hrs.	D. 27 hrs.	D. 27 hrs.	D. 27 hrs.	D. 82 hrs.	D. 82 hrs.	D. 27 hrs.
0.000001	0.2	D. 27 hrs.	D. 82 hrs.	D. 29 hrs.	D. 46 hrs.	D. 82 hrs.	Survived.	D. 30 hrs.

Experiment 7.—The serum (411) for the following test was obtained from a patient, with severe lobar pneumonia, involving both lower lobes. The temperature fell by lysis on the seventh day of disease, reaching normal ten days later. The blood culture yielded *Diplococcus lanceolatus*. The organism, when isolated, was of low virulence for mice, 0.1 c.c. of a twenty-four hour broth culture failing to kill. Before testing the protective power of the patient's serum, the virulence was raised by animal passage until 0.000001 c.c. of a twenty-four hour broth culture was fatal for mice in forty-eight hours.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, syphilis.	Serum 411, Dec. 16, 1911, 3 days before beginning of lysis.	Serum 411, Dec. 19, 1911, during lysis.	Serum 411, Jan. 3, 1912, 14 days after temperature reached normal.
0.1	0.2	—	—	D. 18 hrs.	D. 18 hrs.	—
0.01	0.2	—	—	D. 18 hrs.	D. 24 hrs.	—
0.001	0.2	D. 36 hrs.	D. 36 hrs.	D. 18 hrs.	Survived.	D. 18 hrs.
0.0001	0.2	D. 5 days.	D. 36 hrs.	D. 18 hrs.	D. 54 hrs.	D. 4 days.
0.00001	0.2	D. 24 hrs.	D. 28 hrs.	D. 24 hrs.	D. 36 hrs.	Survived.
0.000001	0.2	D. 36 hrs.	D. 28 hrs.	D. 5 days.	Survived.	Survived.

Experiment 8.—The serum (503) for this experiment was obtained from a typical case of lobar pneumonia, terminating by crisis on the eighth day of the disease. The blood cultures during the course of the disease were negative. The protective power of the serum was tested against two strains of pneumococci; one, pneumococcus X, a stock culture, had received a great number of animal passages; the other, pneumococcus IX, had been isolated from the sputum of the patient and passed through five animals until 0.000001 c.c. of a twenty-four hour broth culture was fatal for mice in forty-eight hours. It was possible to protect mice against infection with both strains by the use of the serum of a horse immunized to a single strain of pneumococcus Neufeld I.

Protection of Mice against Pneumococcus X (Stock Organism).

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Immune horse serum.	Serum 503, Jan. 25, 1912, 5 days before crisis.	Serum 503, Jan. 29, 1912, 24 hours before crisis.	Serum 503, Jan. 30, 1912, day of crisis.	Serum 503, Feb. 14, 1912, 14 days after crisis.
0.1	0.2	—	Survived.	—	D. 20 hrs.	D. 22 hrs.	D. 28 hrs.
0.01	0.2	—	Survived.	—	D. 24 hrs.	D. 28 hrs.	D. 23 hrs.
0.001	0.2	D. 36 hrs.	Survived.	D. 24 hrs.	D. 28 hrs.	D. 36 hrs.	D. 36 hrs.
0.0001	0.2	D. 36 hrs.	Survived.	D. 36 hrs.	D. 36 hrs.	D. 46 hrs.	D. 50 hrs.
0.00001	0.2	D. 36 hrs.	Survived.	D. 36 hrs.	D. 46 hrs.	D. 36 hrs.	D. 50 hrs.
0.000001	0.2	D. 23 hrs.	Survived.	D. 36 hrs.	D. 60 hrs.	D. 36 hrs.	D. 36 hrs.

Protection of Mice against Pneumococcus IX (Organism from Sputum of Patient 503).

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Immune horse serum.	Serum 503, Jan. 25, 1912, 5 days before crisis.	Serum 503, Jan. 29, 1912, 24 hours before crisis.	Serum 503, Jan. 30, 1912, day of crisis.	Serum 503, Feb. 14, 1912, 14 days after crisis.
0.1	0.2	—	—	—	—	—	—
0.01	0.2	—	—	—	D. 30 hrs.	D. 30 hrs.	—
0.001	0.2	D. 48 hrs.	Survived.	D. 30 hrs.	D. 46 hrs.	Survived.	Survived.
0.0001	0.2	D. 30 hrs.	Survived.	D. 48 hrs.	D. 46 hrs.	Survived.	Survived.
0.00001	0.2	D. 46 hrs.	Survived.	D. 48 hrs.	Survived.	Survived.	Survived.
0.000001	0.2	D. 49 hrs.	Survived.	D. 46 hrs.	D. 46 hrs.	Survived.	Survived.

Experiment 9.—The following specimens of sera (501) were obtained from a patient with typical pneumonia of the right upper lobe. There was a pseudo-crisis on the fifth day of the disease, and a true crisis on the following day, after which the temperature remained normal. The pneumococcus used in the tests was isolated from the patient's sputum and the virulence raised by animal passage until 0.000001 c.c. of a twenty-four hour broth culture was fatal in thirty-six hours. The blood culture was negative throughout the disease.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, leg ulcer.	Serum 501, Feb. 19, 1912, 3 days before crisis.	Serum 501, Feb. 21, 1912, 1 day before crisis. Day of pseudocrisis.	Serum 501, Feb. 23, 1912, 1 day after crisis.	Serum 501, Mar. 11, 1912, 16 days after crisis.
0.1	0.2	—	—	—	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.
0.01	0.2	—	—	—	D. 18 hrs.	D. 18 hrs.	D. 18 hrs.
0.001	0.2	D. 36 hrs.	D. 36 hrs.	D. 18 hrs.	D. 36 hrs.	D. 19 hrs.	D. 36 hrs.
0.0001	0.2	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	Survived.
0.00001	0.2	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 48 hrs.	Survived.
0.000001	0.2	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	Survived.

Experiment 10.—The serum (502) for this test was obtained from a patient suffering from a severe attack of lobar pneumonia. The temperature fell by lysis on the eighth day of the disease, reaching normal on the tenth day. The blood cultures were negative. The pneumococcus used for infection of animals was obtained from the patient's sputum, and after several animal passages 0.000001 c.c. of a twenty-four hour broth culture was fatal for mice in thirty-six hours.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, leg ulcer.	Serum 502, Feb. 19, 1912, 6 days before drop in temperature.	Serum 502, Feb. 21, 1912, 3 days before drop in temperature.	Serum 502, Feb. 27, 1912, 1st day of normal temperature.	Serum 502, Mar. 11, 1912, 13 days after normal temperature.
0.1	0.2	—	—	—	—	D. 18 hrs.	D. 18 hrs.
0.01	0.2	—	—	—	—	D. 30 hrs.	D. 18 hrs.
0.001	0.2	—	—	—	—	D. 18 hrs.	D. 30 hrs.
0.0001	0.2	D. 36 hrs.	D. 18 hrs.	D. 22 hrs.	D. 36 hrs.	D. 24 hrs.	D. 22 hrs.
0.00001	0.2	D. 24 hrs.	D. 24 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 18 hrs.
0.000001	0.2	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.	D. 36 hrs.

Experiment 11.—The serum (554) used in this experiment came from a patient with typical pneumonia of the left lower lobe. The temperature fell by crisis on the sixth day of the disease. The blood culture was negative. The pneumococcus was isolated from the sputum and the virulence raised until 0.000001 c.c. was fatal for mice in thirty-six hours.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, leg ulcer.	Serum 554, Mar. 16, 1912, 1 day before crisis.	Serum 554, Mar. 18, 1912, 12 hours after crisis.	Serum 554, Apr. 5, 1912, 17 days after crisis.
0.1	0.2	—	—	—	D. 18 hrs.	D. 18 hrs.
0.01	0.2	—	—	—	D. 28 hrs.	D. 18 hrs.
0.001	0.2	D. 28 hrs.	D. 18 hrs.	D. 18 hrs.	D. 3 days.	D. 3 days.
0.0001	0.2	D. 28 hrs.	D. 18 hrs.	D. 18 hrs.	D. 6 days.	Survived.
0.00001	0.2	D. 18 hrs.	Survived.	D. 28 hrs.	D. 3 days.	Survived.
0.000001	0.2	D. 36 hrs.	Survived.	D. 36 hrs.	Survived.	Survived.

Experiment 12.—The serum (538) for this experiment was obtained from a patient with a moderately severe attack of lobar pneumonia. The temperature fell by crisis on the seventh day of the disease. The blood cultures were negative. The organism used was a pneumococcus obtained from the patient's sputum. The virulence after animal passage was high, and 0.000001 c.c. of a twenty-four hour broth culture was fatal for mice in thirty-six hours.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum, syphilis.	Serum 538, Mar. 18, 1912, 1 day before crisis.	Serum 538, Mar. 21, 1912, 36 hours after crisis.	Serum 538, Apr. 5, 1912, 16 days after crisis.
0.1	0.2	—	—	—	D. 18 hrs.	D. 18 hrs.
0.01	0.2	—	—	—	D. 24 hrs.	D. 18 hrs.
0.001	0.2	—	—	—	D. 24 hrs.	D. 24 hrs.
0.0001	0.2	D. 24 hrs.	D. 24 hrs.	D. 24 hrs.	Survived.	D. 24 hrs.
0.00001	0.2	D. 36 hrs.	D. 24 hrs.	D. 24 hrs.	Survived.	D. 24 hrs.
0.000001	0.2	D. 36 hrs.	D. 24 hrs.	D. 36 hrs.	Survived.	D. 36 hrs.

Experiment 13.—The serum (606) for this experiment was obtained from a patient with a severe attack of lobar pneumonia. The temperature fell by lysis on the eleventh day of the disease, but remained slightly elevated until the seventeenth day. The physical signs in the lungs cleared up slowly. The blood culture showed pneumococcus on the seventh day, and the organisms disappeared from the blood on the tenth day. The pneumococcus, when isolated, killed mice in the higher dilutions in from three to four days.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence: no serum.	Control serum, syphilis.	Serum 606, Apr. 30, 1912, 4 days before beginning of lysis.	Serum 606, May 1, 1912, 3 days before beginning of lysis.	Serum 606, May 3, 1912, 1 day before beginning of lysis.	Serum 606, May 6, 1912, during lysis.	Serum 606, May 10, 1912, 1st day of normal temperature.	Serum 606, May 20, 1912, 10 days after normal temperature.
0.1	0.2	—	D. 40 hrs.	D. 26 hrs.	D. 24 hrs.	D. 24 hrs.	D. 20 hrs.	D. 20 hrs.	D. 48 hrs.
0.01	0.2	D. 42 hrs.	D. 30 hrs.	D. 40 hrs.	D. 26 hrs.	D. 20 hrs.	D. 26 hrs.	D. 48 hrs.	Survived.
0.001	0.2	D. 42 hrs.	D. 24 hrs.	D. 40 hrs.	D. 40 hrs.	D. 40 hrs.	D. 72 hrs.	D. 30 hrs.	Survived.
0.0001	0.2	D. 4 days.	D. 72 hrs.	D. 4 days.	D. 40 hrs.	D. 4 days.	D. 48 hrs.	D. 48 hrs.	Survived.
0.00001	0.2	D. 4 days.	D. 4 days.	D. 40 hrs.	D. 40 hrs.	D. 72 hrs.	D. 80 hrs.	Survived.	D. 3 days.
0.000001	0.2	D. 48 hrs.	D. 40 hrs.	D. 48 hrs.	D. 72 hrs.	D. 30 hrs.	D. 4 days.	D. 4 days.	Survived.

Experiment 14.—The serum (553) for the following test was obtained from a patient with a moderately severe pneumonia of the left lower lobe. There was a pseudocrisis on the fourth day of the disease. The true crisis occurred on the eighth day of the disease. The blood cultures were negative. The virulence of the pneumococcus isolated from the patient's sputum could not be raised successfully, so the same organism was used for infection of animals as in experiment 13.

Quantity of culture in c.c.	Quantity of serum in c.c.	Virulence; no serum.	Control serum. syphilis.	Serum 553. Mar. 5, 1912, 6 days before crisis.	Serum 553, Mar. 7, 1912, 4 days before crisis. Day of pseudo-crisis.	Serum 553. Mar. 11, 1912, day of crisis.	Serum 553. Mar. 26, 1912, 15 days after crisis.
0.1	0.2	—	D. 40 hrs.	—	—	D. 24 hrs.	D. 40 hrs.
0.01	0.2	D. 42 hrs.	D. 30 hrs.	—	—	D. 40 hrs.	D. 20 hrs.
0.001	0.2	D. 42 hrs.	D. 24 hrs.	D. 4 days.	D. 40 hrs.	D. 40 hrs.	D. 5 days.
0.0001	0.2	D. 4 days.	D. 72 hrs.	D. 4 days.	D. 4½ days.	Survived.	D. 40 hrs.
0.00001	0.2	D. 4 days.	D. 4 days.	D. 4½ days.	D. 4 days.	Survived.	Survived.
0.000001	0.2	D. 48 hrs.	D. 4 days.	D. 4 days.	Survived.	Survived.	Survived.

DISCUSSION.

Specimens of sera obtained at various stages of the disease in fourteen patients have been studied. The specimens of sera from ten of these patients were tested against homologous organisms. The sera from three of the cases were tested against a stock organism, Neufeld I, and from one case against an organism isolated from the blood of patient 606. The appearance of protective substances is more evident when sera are tested against homologous organisms. In the ten groups of sera tested against homologous organisms, all but one exhibited at some time the power to protect mice against infection. Of the four groups of sera tested against the stock organisms, three showed at no time any protective power. In experiment 14, the serum obtained on the day of crisis protected against 100 lethal doses of an organism obtained from the blood of patient 606. It has been shown by Neufeld and Haendel⁷ that the immune bodies for various races of typical pneumococci are highly specific, and we have also found this to be the case in our study of the protective power of an immune serum produced from a type strain. It is, therefore, evident that in the present study the results can only be of importance in the cases where homologous organisms were employed. This is shown by experiment 8, in which the same specimens of sera were tested against two strains of pneumococci.

⁷ Neufeld, F., and Haendel, *Arb. a. d. k. Gsndtsamte*, 1910, xxxiv, 293.

one a stock culture and the other an organism isolated from the patient's sputum. At no time did the serum protect against the stock strain, whereas the post-critical sera protected regularly against one thousand times the lethal dose of the homologous organism.

The amount of protection afforded by the pneumonic sera examined has varied in different individuals. In comparison with the potency of sera obtainable by active immunization of larger animals, it has been small. The greatest amount of protection against strains of maximum virulence is seen in experiments 4 and 8. In both these instances it was possible to protect mice with specimens of serum obtained after the crisis against 0.001 of a cubic centimeter of a broth culture of a highly virulent pneumococcus, of which 0.000001 of a cubic centimeter of the same culture was fatal for mice in forty-eight hours, thus affording protection against one thousand times the lethal dose, a value that corresponds favorably with that of some of the commercial immune sera. In other experiments the protective power of the sera tested varied from ten to one hundred times the lethal dose.

The time at which the appearance of immune bodies in the blood was demonstrable varied somewhat. In only four cases was there any protective power demonstrable in the serum obtained before the crisis. In experiment 1 protective substances were present twelve hours before the drop in temperature. Inasmuch as no earlier specimen of serum had been obtained in this instance, it is impossible to say how long these bodies had been present. In experiment 5 serum obtained two days before the temperature fell showed slight protective power, but no greater than control serum from a patient with chronic endocarditis. In two other experiments, 8 and 14, single animals that had received ante-critical sera survived. In seven cases protective substances either appeared for the first time or showed a marked increase in amount at the time of crisis or, in case of lysis, during the period when the symptoms were abating. In some of these instances no specimens of serum were obtained immediately before the crises, so that the immune bodies may have been present twenty-four hours or longer before the disappearance of symptoms. However, in experiment 4 the

specimen of serum obtained twenty hours before the beginning of the critical drop in temperature contained no protective bodies. In two cases the serum taken during the period of defervescence exhibited little or no power of protection, even against homologous strains of pneumococcus, and it was not for some time later, in one case sixteen days, that the presence of protective substances in the blood was demonstrated. The sera of seven patients showed undiminished protective power as long as the individuals were under observation,—from five to twenty days. In five cases there was a drop in the protective power during the period of convalescence. This diminution varied from a slight loss to complete absence of protective substances. In several instances there was a slight return of protective power during late convalescence. It was thought at one time that the disappearance of antibodies might have some connection with the continuance of slight fever after the critical fall of temperature, but the same phenomenon has been observed in patients that remained afebrile after the crisis.

CONCLUSIONS.

These experiments demonstrate that protective substances are usually present in the blood of patients recovering from lobar pneumonia. As a rule, the appearance of protective bodies in the blood, when demonstrable, coincides rather sharply with the period of critical fall in temperature and the disappearance of symptoms. These substances are not present in the blood in any measurable degree before the crisis, but afterward they may reach a considerable concentration. In certain instances, protective substances either become evident some time after the occurrence of the crisis, or cannot be demonstrated at any period of the disease. Experiments in which it is possible to test serum against an homologous strain of pneumococcus yield in the majority of cases evidence of the presence of protective bodies; whereas in those in which stock cultures are used, the serum, as a rule, shows no protection. The development of specific protective substances in the serum of patients with lobar pneumonia suggests that these bodies may play a part in the mechanism of recovery.

THE OCCURRENCE AND VIRULENCE OF PNEUMO-
COCCI IN THE CIRCULATING BLOOD DURING
LOBAR PNEUMONIA AND THE SUSCEPTI-
BILITY OF PNEUMOCOCCUS STRAINS
TO UNIVALENT ANTIPNEUMO-
COCCUS SERUM.*

By A. R. DOCHEZ, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

The suggestion has been made that lobar pneumonia is primarily a blood infection and the local process in the lung is but part of a generalized condition. This view arose from the discovery that the pneumococcus may at times appear in the circulating blood of persons suffering from pneumonia. Reports in recent years have varied considerably as to the frequency with which it is possible to obtain the pneumococcus from the blood. Some observers have obtained positive cultures in from 20 to 40 per cent. of cases examined and, as they were the more severe cases, they think the presence of the pneumococcus in the blood is of bad prognostic import. Others, claiming a more suitable technique, have found the pneumococcus in the blood during pneumonia with great constancy, and attach very little prognostic significance to its presence. It has been stated that the organisms may be present in considerable numbers, especially in fatal cases, and Fränkel¹ mentions a case in which cultures from the blood during life yielded 2,700 colonies per cubic centimeter of blood.

In thirty-seven cases of lobar pneumonia studied in this hospital, the pneumococcus was not found constantly present in the blood. In those cases in which a positive culture was obtained, the course of the infection was invariably severe and in most instances terminated fatally. Studies of inflammation have shown that micro-

* Received for publication, August 1, 1912.

¹ Fränkel, A. A., *Internationale Beiträge zu innere Medizin*, Berlin, 1902, ii, 103.

organisms in the early stages of the process are taken up by the lymphatics and are passed through them to the adjacent lymph glands. As the inflammatory process progresses, the lymph glands swell, the lymphatic sinuses become packed with cells, and the gland in this way functions as a bacterial filter. That pneumococci may pass the barrier of lymphatic glands in the early stages of pneumonia and in this manner reach the circulation is not unlikely. The majority, however, are probably destroyed rapidly and never develop sufficiently to be readily demonstrable in cultures from the blood. Some, having gained access, may grow slowly for a time and then disappear, or as the body defenses become weakened by intoxication, they may multiply rapidly and cause the death of the individual. In certain cases of pneumonia, it seems that the pneumococci either invade the blood in large numbers or else, having arrived there, grow so actively that death ensues from septicemia before any considerable area of lung has become involved. The possibility must be borne in mind that invasion of the blood may not occur by way of lymphatic channels alone, but that vessel walls may become so injured by bacterial products that direct extension of the infection through them into the blood stream arises. However the organisms may get into the blood, the important question is whether, once having gotten there, they are able to develop. As long as the number of pneumococci in the blood is inconsiderable, probably no great harm results, but as soon as they begin to grow actively, the condition is a far more serious menace to the life of the individual than the local process in the lung.

CASES STUDIED.

In the following cases blood was obtained for the culture by venous puncture. Usually about 5 cubic centimeters of blood were added to a flask containing from 75 to 125 cubic centimeters of bouillon prepared from fresh meat, to which from 20 to 25 cubic centimeters of normal sodium hydrate per liter had been added. *Pneumococcus* grows luxuriantly and without difficulty in this medium. Repeated cultures were made from the same individual in many cases and in some instances solid cultures were prepared in

order to determine the number of pneumococci per cubic centimeter of blood.

Among the thirty-seven cases detailed in table I, pneumococci were present in the blood at some stage of the disease in eighteen instances, approximately in 50 per cent. of the cases. Of these eighteen cases, fourteen, or 77 per cent., were fatal, whereas four cases recovered. Of the nineteen cases in which blood cultures were negative, fifteen, or 79 per cent., recovered and four died. The patients were chiefly persons of a low social class, and many of them were habitual users of alcohol. The total mortality was unusually high, 48 per cent., and of the total number of fatal cases the pneumococcus was isolated from the blood in 77 per cent. In four instances the first blood culture was negative and subsequent cultures at a later stage of the disease showed the presence of the pneumococcus. In the majority of instances, where a positive culture was obtained, the number of bacteria per cubic centimeter of blood was estimated from solid cultures. The counts, as a rule, were extraordinarily high shortly before or at the time of death, showing thousands of colonies. The extent of the local disease was not always comparable to the severity of blood invasion, and in two instances seemed inconspicuous. In two fatal cases where low bacterial counts were obtained from the blood, the local process in the lung spread with great rapidity. In case XV there were 10,000 pneumococci per cubic centimeter of blood on the fourth day of the disease. The highest count obtained was in case XXXVII, being 65,000 colonies per cubic centimeter of blood obtained at the time of death on the eighth day of disease. Of the four fatal cases without positive blood cultures, one died of nephritis after the subsidence of the pneumonia, and three showed a spreading infection in the lungs. Of the nineteen cases that recovered, four had pneumococci in the blood, and in fifteen the blood cultures were negative. The course of the disease in the four cases with positive blood cultures was severe in three instances and moderate in one. In two of the cases the pneumococcus isolated from the blood was avirulent, in one it killed animals slowly, and in the other was of high virulence. In recovered cases XXXIV and XXXVI, successive counts of the bacteria in the blood were made. The maximum count in case

XXXIV was sixty colonies per cubic centimeter on the fifth day of the disease. On the sixth day the count had dropped to less than one colony per cubic centimeter, and on the seventh day, the day of lysis, the culture was negative. The organism isolated in this case was highly virulent and the course of the disease was complicated by empyema. In case XXXVI the maximum count per cubic centimeter of blood was less than one colony on the eighth day of the disease. On the tenth day, the day of lysis, the blood culture was negative. Of the fifteen recovered cases with negative blood cultures, the course of the disease was usually not very severe and there seemed to be little tendency for the disease to spread beyond the limits of the lobe of lung originally infected.

The object of this study has been to emphasize the infectious side of lobar pneumonia. The two most striking characteristics of pneumococcus infection of the lung are the tendency of the local lesion to spread and the tendency to serious invasion of the blood by the bacteria. The local process usually develops until it meets the opposition to further extension afforded by the pleural membrane. The opinion is quite generally prevalent among clinicians that the extent of involvement of lung tissue in pneumonia bears no relation to the severity of the disease. In support of this assumption, severe and rapidly fatal cases are cited in which simply one upper lobe is involved, or in which the pneumonic process lies deep within a lobe and is difficult to recognize clinically. The cause of death is ascribed to failure of the circulation dependent upon the unusually severe intoxication attending such infections. Careful study of the blood in these instances would probably reveal a septicemic condition sufficient to account for the fatal result, regardless of the local disease in the lung. Considerable doubt exists as to whether any previously strong individual dies from the intoxication arising from a small area of pneumonic consolidation of the lung. Certainly in this hospital no instance of death occurred when the process was confined to a single lobe and where there was no bacterial invasion of the blood. Death in pneumonia seems therefore to depend chiefly upon an extensive invasion of the blood by the organisms, or upon a rapid and extensive spread of the local lesion in the lung.

XXVII	535	34 yrs.	10th	2 dys. 18 hrs.	glucose agar, glucose agar	Positive	—	7th	A	<p>eleventh day of the disease. A bouillon culture on the sixth day of disease yielded pure pneumococcus. Plates on the eighth day showed 2,900 colonies of mixed pneumococcus and <i>Staphylococcus albus</i> per c.c. of blood. On the tenth day there were 245 colonies of pure pneumococcus, and on the eleventh day 15,000 colonies per c.c. of blood. There was severe infection involving first the left</p>
			11th			Positive	—			
			4th	3 dys.	Bouillon	Positive	Died			

VIRULENCE FOR WHITE MICE OF PNEUMOCOCCI ISOLATED FROM THE BLOOD DURING PNEUMONIA.

Frequent attempts have been made to correlate the progress of lobar pneumonia with variations in the virulence of the pneumococcus during the course of the infection. A satisfactory assumption would seem to be that the more virulent the pneumococcus concerned, the more severe the character of the disease. Aside from the fact that virulence must be tested upon a different species of animal, the varying efficiency of the resources of the host, in the struggle for mastery between parasite and host, makes it impossible to predict the outcome of an infection from a knowledge of the virulence alone. Studies in the variation of virulence of the pneumococcus have been made largely upon organisms obtained from the sputum. In general, the results have been variable, but there seems to be no definite relationship between the virulence of the organism as tested experimentally and the clinical course of the disease, nor can the critical termination of pneumonia be attributed to a sudden loss of virulence of the infecting pneumococcus.²

In the course of the bacteriological examination of the blood of patients described above, the virulence of the first cultures for white mice was regularly determined. No observations were made upon organisms obtained from the sputum because of the possible influence that the manipulations necessary for isolation may have upon the character of the organism. The custom has been to use the blood culture after twenty-four hours' growth, a period that has always permitted vigorous development. Varying dilutions of this culture with meat bouillon were made and injected intraperitoneally into white mice. The results are presented in table II.

Of the eighteen strains of pneumococcus isolated from the blood, thirteen were of extremely high original virulence for mice, doses of 0.00001 of a cubic centimeter or less of a twenty-four hour broth culture killing mice in less than thirty-six hours. Twelve of the individuals from whom highly virulent cultures were obtained died, and one recovered, convalescence being complicated by empyema. Three strains of medium virulence were obtained. One of these failed to kill mice in doses of 0.01 of a cubic centimeter, and two

² Luetscher, J. A., *Jour. Infect. Dis.*, 1911, ix, 287.

TABLE II.

Number of case.	Number of record.	Day of disease.	Recovery or death.	Virulence of culture.	
I	423	5th	Died	0.01 c.c. D. 20 hrs. 0.0001 c.c. D. 20 hrs. 0.000001 c.c. D. 24 hrs.	
II	428	4th	Died	0.1 c.c. D. 40 hrs. 0.01 c.c. D. 3 dys. 0.0001 c.c. D. 3 dys. 0.000001 c.c. D. 6 dys.	
III	438	2d	Died	0.01 c.c. D. 20 hrs. 0.0001 c.c. D. 20 hrs. 0.000001 c.c. D. 20 hrs.	
IV	119	7th	Recovered	1.0 c.c. D. 36 hrs. 0.5 c.c. Survived.	
V	453	Autopsy	Died	0.5 c.c. D. 18 hrs. 0.1 c.c. D. 18 hrs. 0.01 c.c. Survived. 0.0001 c.c. Survived.	
VI	472	5th	Died	0.01 c.c. D. 20 hrs. 0.0001 c.c. Survived. 0.000001 c.c. D. 36 hrs.	
VII	480	6th	Died	0.01 c.c. D. 20 hrs. 0.0001 c.c. D. 20 hrs. 0.000001 c.c. D. 22 hrs.	
VIII	411	7th	Recovered	2.0 c.c. D. 14 hrs. 0.1 c.c. Survived. 0.01 c.c. Survived.	
IX P. mucosus	484	7th	Died	0.01 c.c. D. 11 hrs. 0.0001 c.c. D. 17 hrs. 0.000001 c.c. D. 21 hrs.	
X	487	3d	Died	0.0001 c.c. D. 20 hrs. 0.00001 c.c. D. 21 hrs. 0.000001 c.c. D. 24 hrs.	
XI	525	4th	Died	0.0001 c.c. D. 18 hrs. 0.00001 c.c. D. 24 hrs. 0.000001 c.c. D. 24 hrs.	
XII	533	6th	Died	0.0001 c.c. D. 24 hrs. 0.00001 c.c. D. 18 hrs. 0.000001 c.c. D. 24 hrs.	
XIII	535	6th	Died	0.0001 c.c. D. 28 hrs. 0.00001 c.c. D. 28 hrs. 0.000001 c.c. D. 28 hrs.	
XIV	551	2d	Recovered, empyema	0.0001 c.c. D. 18 hrs. 0.00001 c.c. D. 24 hrs. 0.000001 c.c. D. 26 hrs.	

TABLE II (*Continued*).

Number of case.	Number of record.	Day of disease.	Recovery or death.	Virulence of culture.
XV P. <i>mnucosus</i>	544	11th	Died	0.0001 c.c. D. 36 hrs. 0.00001 c.c. D. 36 hrs. 0.000001 c.c. D. 36 hrs.
XVI	447	7th	Died	0.0001 c.c. D. 22 hrs. 0.00001 c.c. D. 24 hrs. 0.000001 c.c. D. 24 hrs.
XVII	606	7th	Recovered	0.1 c.c. D. 36 hrs. 0.01 c.c. D. 3 dys. 0.0001 c.c. D. 4 dys. 0.00001 c.c. D. 5 dys. 0.000001 c.c. D. 3 dys.
XVIII	617	6th	Died	0.0001 c.c. D. 24 hrs. 0.00001 c.c. D. 24 hrs. 0.000001 c.c. D. 24 hrs.

killed animals in the higher dilutions only after considerable periods of time. Two patients, from whom these organisms were obtained, died, and one recovered without complications. Relatively avirulent strains were isolated from the blood in two cases, and in both instances the individuals recovered without complications.

The number of cases is entirely too few to permit any general deductions concerning the relation of the virulence of the pneumococcus to the course of the disease. As far as the indication goes, it would seem probable that individuals having an avirulent organism in the blood stand a better chance of recovery than those in whom the organism is of high virulence. That the latter quality necessarily indicates a fatal issue is disproven by case XIV, in which the individual recovered in spite of the presence of a considerable number of highly virulent pneumococci (sixty per cubic centimeter of blood). It is of some interest that most of the organisms isolated possessed a virulence comparable with that of the highly virulent laboratory strains. If the organisms obtained from the blood represent descendants of previous inhabitants of the mouth, there can be very little doubt that there has been a marked accession of virulence during the period of growth in the lungs and blood.

SUSCEPTIBILITY OF BLOOD AND SPUTUM STRAINS OF PNEUMOCOCCUS
TO THE ACTION OF UNIVALENT ANTIPNEUMOCOCCUS SERUM.

Specific immune sera have been prepared by a number of investigators and have been used for the study of experimental pneumococcus infection in animals, and also in the treatment of lobar pneumonia and other pneumococcus infections in man. In animals it has been possible to prevent infection with pneumococcus when immune serum is given simultaneously with a lethal dose of pneumococci, and also to inhibit development of the organism when serum is given within three or four hours after infection. The effect of serum therapy upon lobar pneumonia in man has not been striking. Some observers have noticed a beneficial action and think they have noticeably affected the course of the disease. Study of the mortality statistics, however, of serum treated cases shows no appreciable difference in the death rate of individuals so treated and those untreated. With a better technique of serum administration, it may be possible to develop more striking effects, and with this end in view Neufeld³ has urged the intravenous injection of large quantities of immune serum.

In this hospital, during the past year, an antipneumococcus serum of high potency has been developed by immunization of the horse with large doses of living virulent organisms. The serum was prepared by immunizing an animal against a single strain of pneumococcus, a typical organism of constant high virulence obtained from Professor Neufeld. The protective action of this univalent serum was tested against all strains of pneumococcus isolated from patients in the hospital. The organisms in most instances came from individuals suffering from lobar pneumonia. Pneumococci⁴ have not as yet been divided into clearly defined biological groups, though it is known that a serum derived from a certain typical strain may fail to protect animals against infection with other strains that are morphologically and culturally characteristic. The following experiments were performed in order to determine the number of races of pneumococci against which a univalent serum would afford protection. White mice were used for experimental infection.

³ Neufeld, F., and Händel, L., *Berl. klin. Wchnschr.*, 1912, xlix, 680.

⁴ Neufeld, F., and Händel, *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 159.

TABLE III.

Number of organism.	Source.	Virulence.		Serum protection.		
		Amount of culture.	Result.	Amount of culture.	Amount of serum.	Result.
II	Lobar pneumonia blood	0.0001 c.c.	D. 24 hrs.	0.0001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 24 hrs.	0.00001 c.c.	0.2 c.c.	D. 24 hrs.
		0.000001 c.c.	D. 24 hrs.	0.000001 c.c.	0.2 c.c.	Survived.
III	Broncho-pneumonia sputum	0.00001 c.c.	D. 24 hrs.	0.1 c.c.	0.2 c.c.	D. 24 hrs.
		0.000001 c.c.	D. 24 hrs.	0.01 c.c.	0.2 c.c.	D. 24 hrs.
				0.0001 c.c.	0.2 c.c.	Survived.
IV	Lobar pneumonia blood	0.0001 c.c.	D. 40 hrs.	0.1 c.c.	0.2 c.c.	Survived.
		0.00001 c.c.	D. 48 hrs.	0.01 c.c.	0.2 c.c.	Survived.
		0.000001 c.c.	D. 40 hrs.	0.001 c.c.	0.2 c.c.	Survived.
V	Lobar pneumonia blood	0.0001 c.c.	D. 18 hrs.	0.0001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 18 hrs.	0.00001 c.c.	0.2 c.c.	D. 24 hrs.
		0.000001 c.c.	D. 24 hrs.	0.000001 c.c.	0.2 c.c.	D. 24 hrs.
VII	Lobar pneumonia sputum	0.0001 c.c.	D. 42 hrs.	0.1 c.c.	0.2 c.c.	Survived.
		0.00001 c.c.	D. 42 hrs.	0.01 c.c.	0.2 c.c.	Survived.
		0.000001 c.c.	D. 42 hrs.	0.001 c.c.	0.2 c.c.	Survived.
IX	Lobar pneumonia sputum	0.0001 c.c.	D. 30 hrs.	0.001 c.c.	0.2 c.c.	Survived.
		0.00001 c.c.	D. 46 hrs.	0.0001 c.c.	0.2 c.c.	Survived.
		0.000001 c.c.	D. 49 hrs.	0.00001 c.c.	0.2 c.c.	Survived.
X	Lobar pneumonia blood	0.0001 c.c.	D. 24 hrs.	0.1 c.c.	0.2 c.c.	Survived.
		0.00001 c.c.	D. 24 hrs.	0.01 c.c.	0.2 c.c.	Survived.
		0.000001 c.c.	D. 40 hrs.	0.001 c.c.	0.2 c.c.	Survived.
S. E. I	Endocarditis blood	0.001 c.c.	D. 22 hrs.	0.01 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 42 hrs.	0.001 c.c.	0.2 c.c.	D. 18 hrs.
		0.000001 c.c.	D. 22 hrs.	0.0001 c.c.	0.2 c.c.	D. 22 hrs.
XIV	Meningitis spinal fluid	0.3 c.c.	D. 20 hrs.	0.3 c.c.	0.2 c.c.	D. 20 hrs.
		0.1 c.c.	D. 24 hrs.	0.1 c.c.	0.2 c.c.	D. 20 hrs.
		0.01 c.c.	Survived.	0.01 c.c.	0.2 c.c.	Survived.
XV	Lobar pneumonia blood	0.0001 c.c.	D. 18 hrs.	0.0001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 18 hrs.	0.00001 c.c.	0.2 c.c.	D. 18 hrs.
		0.000001 c.c.	D. 18 hrs.	0.000001 c.c.	0.2 c.c.	D. 18 hrs.
XVI	Lobar pneumonia blood	0.0001 c.c.	D. 5 dys.	0.001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 24 hrs.	0.0001 c.c.	0.2 c.c.	D. 4 dys.
		0.000001 c.c.	D. 36 hrs.	0.00001 c.c.	0.2 c.c.	D. 5 dys.
				0.000001 c.c.	0.2 c.c.	Survived.
XVII	Lobar pneumonia sputum	0.0001 c.c.	D. 36 hrs.	0.0001 c.c.	0.2 c.c.	D. 36 hrs.
		0.00001 c.c.	D. 36 hrs.	0.00001 c.c.	0.2 c.c.	D. 36 hrs.
		0.000001 c.c.	D. 36 hrs.	0.000001 c.c.	0.2 c.c.	D. 36 hrs.
XIX P. PNEUMOSUS	Lobar pneumonia blood	0.0001 c.c.	D. 18 hrs.	0.0001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 26 hrs.	0.00001 c.c.	0.2 c.c.	D. 27 hrs.
		0.000001 c.c.	D. 36 hrs.	0.000001 c.c.	0.2 c.c.	D. 27 hrs.

TABLE III (Continued).

Number of organism.	Source.	Virulence.		Serum protection.		
		Amount of culture.	Result.	Amount of culture.	Amount of serum.	Result.
XX	Lobar pneumonia blood	0.0001 c.c.	D. 20 hrs.	0.0001 c.c.	0.2 c.c.	D. 20 hrs.
		0.00001 c.c.	D. 20 hrs.	0.00001 c.c.	0.2 c.c.	D. 20 hrs.
		0.000001 c.c.	D. 20 hrs.	0.000001 c.c.	0.2 c.c.	D. 20 hrs.
XXI	Lobar pneumonia sputum	0.0001 c.c.	D. 24 hrs.	0.0001 c.c.	0.2 c.c.	D. 3 dys.
		0.00001 c.c.	D. 24 hrs.	0.00001 c.c.	0.2 c.c.	D. 3 dys.
		0.000001 c.c.	D. 36 hrs.	0.000001 c.c.	0.2 c.c.	D. 3 dys.
XXII	Lobar pneumonia blood	0.0001 c.c.	D. 18 hrs.	0.0001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 24 hrs.	0.00001 c.c.	0.2 c.c.	D. 24 hrs.
		0.000001 c.c.	D. 24 hrs.	0.000001 c.c.	0.2 c.c.	D. 18 hrs.
XXIII	Lobar pneumonia blood	0.0001 c.c.	D. 24 hrs.	0.0001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 18 hrs.	0.00001 c.c.	0.2 c.c.	D. 24 hrs.
		0.000001 c.c.	D. 24 hrs.	0.000001 c.c.	0.2 c.c.	D. 24 hrs.
XXIV	Lobar pneumonia blood	0.0001 c.c.	D. 28 hrs.	0.001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 28 hrs.	0.0001 c.c.	0.2 c.c.	Survived.
		0.000001 c.c.	D. 28 hrs.	0.00001 c.c.	0.2 c.c.	Survived.
XXV	Lobar pneumonia blood	0.0001 c.c.	D. 18 hrs.	0.1 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 24 hrs.	0.01 c.c.	0.2 c.c.	D. 3 dys.
		0.000001 c.c.	D. 26 hrs.	0.001 c.c.	0.2 c.c.	Survived.
XXVI <i>P. mucosus</i>	Lobar pneumonia lung	0.0001 c.c.	D. 26 hrs.	0.0001 c.c.	0.2 c.c.	D. 26 hrs.
		0.00001 c.c.	D. 24 hrs.	0.00001 c.c.	0.2 c.c.	D. 36 hrs.
		0.000001 c.c.	D. 36 hrs.	0.000001 c.c.	0.2 c.c.	D. 36 hrs.
XXVII	Lobar pneumonia blood	0.0001 c.c.	D. 22 hrs.	0.1 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 24 hrs.	0.01 c.c.	0.2 c.c.	Survived.
		0.000001 c.c.	D. 24 hrs.	0.001 c.c.	0.2 c.c.	Survived.
XXVIII	Lobar pneumonia blood	0.0001 c.c.	D. 4 dys.	0.1 c.c.	0.2 c.c.	D. 3 dys.
		0.00001 c.c.	D. 5 dys.	0.01 c.c.	0.2 c.c.	Survived.
		0.000001 c.c.	D. 3 dys.	0.001 c.c.	0.2 c.c.	Survived.
XXIX	Lobar pneumonia blood	0.0001 c.c.	D. 24 hrs.	0.0001 c.c.	0.2 c.c.	D. 18 hrs.
		0.00001 c.c.	D. 24 hrs.	0.00001 c.c.	0.2 c.c.	D. 24 hrs.
		0.000001 c.c.	D. 24 hrs.	0.000001 c.c.	0.2 c.c.	D. 24 hrs.

Wherever the original virulence of the pneumococcus was low, it was raised by successive animal passages until a high degree of virulence was reached. Twenty-four hour broth cultures made from the heart's blood of passage animals were used for infection. The protective dose of serum and given quantities of diluted culture

were injected simultaneously into the peritoneal cavity. Protection was deemed complete when animals survived inoculation longer than five days (table III).

A univalent serum developed by immunization against pneumococcus N-I was tested for protection against twenty-three strains of pneumococcus. Of these organisms two belonged to the group that is commonly designated as *Pneumococcus* or *Streptococcus mucosus*; one was an organism isolated from the spinal fluid that had certain characteristics belonging to the pneumococcus, and other features that suggested a relationship to streptococcus; and another organism was one obtained from the blood in a case of infectious endocarditis. The latter on isolation resembled streptococcus, but after several passages through animals, acquired many of the characters of pneumococcus. The remaining nineteen strains were all typical pneumococci obtained either from the blood or sputum of patients with pneumonia. Out of fourteen strains of unquestionable pneumococci obtained by blood culture, the univalent serum gave some degree of protection against eight, about 57 per cent. Of five sputum strains, four were susceptible to the protective action of the serum. Of the total number of twenty-three strains against which the serum was tested, including two strains of *Pneumococcus mucosus* and two organisms of doubtful identity, it was proved that protective antibodies were present in twelve instances, slightly more than 50 per cent. The amount of protection afforded by the serum against these strains varied from a grade practically equal to that found when pneumococcus N-I was used for infection to protection against a single lethal dose, or in one instance to simple prolongation of the period of life of the infected animals. In eight instances the serum protected against 1,000 or more lethal doses, and in four the protection was of a lower grade. An antipneumococcus serum, derived by immunization of animals with a certain single type strain of pneumococcus, therefore protects against a considerable proportion of the races ordinarily met with in lobar pneumonia.

If such a univalent serum is used therapeutically in human pneumonia, one knows beforehand that one half or more of the individuals treated must derive no benefit from its use, because of the probable insusceptibility of many of the infecting organisms to the pro-

tective action of the serum. It is not unlikely that if a multiplicity of strains are used indiscriminately in the preparation of an immune serum, the degree of immunity to any single strain would be proportionately low. In view of this supposition, further studies of the biological relationship of different strains of pneumococcus are being made in order to determine the widest range of protection that can be obtained by the use of a minimal number of distinct races. Should it be found that a reasonably small number of strains would yield protective bodies for a large percentage of the varieties of pneumococcus ordinarily met with, it might be possible to immunize single animals to individual strains and in this way obtain sera of the highest protective value for suitable cases. In human beings a fairly rapid test of the susceptibility of the organism concerned could be made, and in this way a serum could be selected for treatment which would give the greatest probability of success.

SUMMARY.

Studies of the bacteriology of the blood were made in thirty-seven cases of lobar pneumonia. The pneumococcus was isolated from the blood in approximately 50 per cent. of the cases studied. The course of infection in individuals with pneumococcus in the blood was more severe than in those in which no organism could be cultivated from the blood. 77 per cent. of the patients with positive blood cultures died, and 79 per cent. of patients with negative blood cultures recovered. In fatal instances of pneumonia, where the pneumococcus was found in the blood, the number of organisms per cubic centimeter of blood was very high in the last stage of the disease. In individuals dying of pneumonia without blood infection, the disease was characterized by a rapid spread of the local process in the lungs. It is not unlikely that the symptoms of collapse, developing on the fifth or sixth day of lobar pneumonia, are often the expression of serious invasion of the blood by the pneumococcus. In other instances, they mark an extension of the local process in the lungs.

Strains of pneumococcus isolated from the blood of patients with lobar pneumonia were usually of high animal virulence. In a few instances where the organism isolated from the blood was of low virulence for animals, the patients recovered.

The protective power of a univalent antipneumococcus serum was tested against nineteen strains of typical pneumococcus and against four strains of closely allied organisms. The serum manifested some degree of protection against twelve out of nineteen strains of typical pneumococci. No protection was observed against the atypical organisms. In eight instances the degree of protection obtained was high, in three low, and in one there occurred only a prolongation of the period of life of the inoculated animal.

COAGULATION TIME OF THE BLOOD IN LOBAR PNEUMONIA.*

By A. R. DOCHEZ, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)

Observation of the variation in time within which the blood coagulates when removed from its normal surroundings is of importance in the study of certain diseases, and accurate determination of the factors involved in producing any variation in the normal coagulation time of the blood would, no doubt, throw some light upon the nature of the pathological processes concerned. A large number of methods designed to estimate the time of coagulation under normal and pathological conditions have been devised. Many of these methods give fairly accurate results in the hands of their originators, but the time of normal coagulation differs considerably when determined by different methods, so that comparison of one observer's results with those of another is unsatisfactory.

Morowitz¹ has devoted much study to the relation of different elements and to the sequence of events in the process of blood coagulation. The most satisfactory and adequate explanation of the phenomenon, however, has recently been offered by Howell,² who has carefully traced the various stages in the reaction, which finally results in the conversion of fibrinogen into fibrin. All the elements necessary for coagulation are present in the circulating blood. The inception of the process is prevented, however, by the presence of a substance, antithrombin, which binds the prothrombin, thus preventing its activation within the vessels by the calcium salts present. When shed blood is exposed to tissue juices or foreign surfaces, a body designated thromboplastin, originating either from tissue juices or the disintegration of certain cellular elements in the blood, develops. Thromboplastin, by its ability to combine with antithrombin, frees the previously bound prothrombin. This immediately reacts with the calcium salts of the blood with the liberation of active thrombin, which then precipitates the fibrinogen as fibrin. This explanation emphasizes the probability that failure of the coagulative power, or changes in the coagulation time of the

* Received for publication, August 1, 1912.

¹ Morowitz, P., *Handbuch der biochemischen Arbeitsmethoden*, 1911, v, 223.

² Howell, W. H., *Am. Jour. Physiol.*, 1911-12, xxix, 187.

blood, are dependent upon variation in some one or more of the factors described. Quantitative estimation of the substances concerned is possible and would yield more valuable results than simple determination of the coagulation time, even were the methods for the latter observation uniform. Whipple^{*} has recently applied such a procedure to the study of certain diseases and urges a classification of conditions associated with hemorrhage upon the basis of the specific element responsible for the change in coagulative power.

In the present paper are given the results obtained from a study of the coagulation time of the blood in a number of cases of lobar pneumonia. On account of the large amount of time required, quantitative estimations of the thrombin, antithrombin, and fibrinogen were not made, so that it is possible to explain only by inference the prolongation of the coagulation time observed during the acute stage of pneumonia.

METHOD.

The method used for determination of the coagulation time was a comparatively simple one. An attempt was made to guard against certain obvious sources of error. A fairly large quantity of blood, about seven cubic centimeters, was used for each determination in order that the proportion of volume to the surface exposed to contact with foreign substances might be as great as possible.

The blood was obtained by rapidly filling an all glass syringe, having a needle of large calibre, with blood directly from the median basilic vein of the patient. A stoppered glass tube was then filled from the syringe to within about two centimeters of the top. The tube had previously been inserted through a rubber stopper into a large glass cylinder containing water kept at 38° C. The average time for the complete operation was about forty-five seconds. The apparatus was then carried to a room kept constantly at 38° C., and after the lapse of some minutes the tube was carefully rotated every thirty seconds. The time of coagulation was measured from the period at which half the quantity of blood had been withdrawn from the vein to the point at which the blood failed upon rotation to flow from one end of the tube to the other. Great care was taken to prevent the presence of air bubbles upon the upper surface of the blood, as these accelerate the formation of a clot at this point. All glassware with which the blood came in contact was maintained as nearly as possible at body temperature and was kept scrupulously clean. The specimen of blood was taken by venous puncture in order to prevent contact with lacerated tissue.

It had been assumed, that if prolongation of the coagulation time occurred, such a condition would probably be dependent upon an

^{*} Whipple, G. H., *Arch. Int. Med.*, 1912, ix 365.

excess of antithrombin. If such were the case, the ordinary method of obtaining drops of blood from a skin puncture would expose it to contamination with thromboplastin, and inasmuch as the excess of antithrombin might be very slight, a sufficient quantity of thromboplastic substances might be absorbed during the formation of a small drop to mask entirely the presence of a slight excess of antithrombin. It is a well known fact that successive drops from the same skin puncture clot with increasing rapidity. This can hardly be looked upon as comparable to the shortening of coagulation time which occurs during hemorrhage. The blood while in the vessels shows no tendency to clot, and the time within which it coagulates when removed depends upon the variety and nature of external conditions, so that different methods in the main give variable results. No special advantages are claimed for the method used in this study. It is possible for the same individual to obtain comparable results if variations in technique are carefully guarded against.

The idea has become current that the coagulation time of the blood is shortened during the acute stage of lobar pneumonia. This opinion has developed in spite of the observations of Hayem,⁴ Pye-Smith,⁵ and Coleman,⁶ that the clotting time is prolonged in pneumonia. Cohen,⁷ in a study of six pneumonia patients, found the average time slightly shortened. The formation of white clots in the heart and vessels of patients dying of lobar pneumonia is a common observation at autopsy. In this hospital a marked sedimentation of the red cells, with the formation of a buff coat, occurred when tubes of blood obtained from pneumonia patients were allowed to stand. Such observations are infrequent in the case of normal blood and suggest that the coagulation time of the blood in pneumonia is sufficiently delayed to allow a settling of the heavier cellular elements.

In the course of some recent experiments in which rabbits were infected with massive doses of pneumococcus, the fact was noted that when the animals died, after the lapse of only a few hours, the blood had already become practically incoagulable. These ob-

⁴ Hayem, G., *Du sang et de ses altérations anatomiques*, Paris, 1889, 323.

⁵ Pye-Smith, P. H., Allbutt and Rolleston, *System of Medicine*, 1898, v, 91.

⁶ Coleman, C. J., *Biochem. Jour.*, 1907, ii, 184.

⁷ Cohen, M. S., *Arch. Int. Med.*, 1911, viii, 684, 820.

servations seemed to indicate the probability of a prolongation of coagulation time during lobar pneumonia. The following cases demonstrate in most instances a definite increase in the clotting time of the blood during the acute stage of lobar pneumonia with a gradual return to normal during the period of convalescence.

Case 1.—411. Severe infection. Recovered. Lysis on the ninth day of disease.

Dec. 16, 1911, three days before lysis.	Dec. 19, 1911, dur- ing lysis.	Jan. 3, 1912, tem- perature normal.
Coagulation time: 10 min.	11 min., 10 sec.	6 min., 30 sec.

Case 2.—418. Mild infection. Recovered. Lysis on the fourth day of disease.

Dec. 24, 1911, two days before lysis.	Dec. 29, 1911, one day after lysis.	Jan. 8, 1912, tem- perature normal.
Coagulation time: 16 min.	10 min.	7 min.

Case 3.—409. Severe infection. Died on the fourth day of disease.

Jan. 4, 1912, second
day of disease.
Coagulation time:
17 min., 10 sec.

Case 4.—413. Severe infection. Died on the fourth day of disease.

Jan. 7, 1912, third
day of disease.
Coagulation time:
13 min.

Case 5.—423. Severe infection. Died on the seventh day of disease.

Jan. 14, 1912, fifth day of disease.	Jan. 16, 1912, two hours before death.
Coagulation time: 15 min.	18 min.

Case 7.—454. Mild infection. Recovered. Crisis on the third day of disease.

Jan. 21, 1912, day of crisis.	Jan. 28, 1912, seven days after crisis.
Coagulation time: 12 min.	10 min., 30 sec.

Case 8.—439. Severe infection. Died on the tenth day of disease.

Jan. 22, 1912, sixth day of disease.	Jan. 25, 1912, ninth day of disease.
Coagulation time: 11 min.	12 min.

Case 9.—438. Severe infection. Died on the fourth day of disease.

Jan. 24, 1912, second day of disease.

Coagulation time:
9 min.

Case 10.—503. Moderately severe infection. Crisis on the seventh day of disease.

Jan. 25, 1912, four days before crisis.	Jan. 29, 1912, one day before crisis.	Jan. 30, 1912, day of crisis.	Feb. 14, 1912, two weeks after crisis.
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Coagulation time: 11 min., 45 sec.	12 min.	9 min.	9 min.
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Case 11.—460. Severe infection. Died on the fifth day of disease.

Feb. 2, 1912, third day of disease.	Feb. 4, 1912, fifth day of disease.
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Coagulation time: 8 min., 45 sec.	11 min., 30 sec.
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Case 12.—472. Severe infection. Died on the fifth day of disease.

Feb. 12, 1912, third day of disease.	Feb. 14, 1912, fifth day of disease.
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Coagulation time: 13 min., 30 sec.	12 min., 45 sec.
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Case 13.—497. Moderately severe infection. Recovered. Crisis on the seventh day of disease.

Feb. 13, 1912, two days before crisis.	Feb. 15, 1912, day of crisis.	Feb. 18, 1912, three days after crisis.
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Coagulation time: 14 min., 15 sec.	11 min., 30 sec.	8 min., 30 sec.
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Case 14.—484. Severe infection. Died on the eighth day of disease.

Feb. 19, 1912, third day of disease.	Feb. 23, 1912, seventh day of disease.
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Coagulation time: 9 min., 15 sec.	13 min., 30 sec.
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Case 15.—502. Severe infection. Recovered. Crisis on the eighth day of disease.

Feb. 19, 1912, seven days before crisis.	Feb. 21, 1912, five days before crisis.	Feb. 27, 1912, day of crisis.	March 11, 1912, thirteen days after crisis.
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Coagulation time: 11 min.	9 min., 15 sec.	7 min., 30 sec.	7 min., 30 sec.
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Case 16.—501. Moderately severe infection. Recovered. Crisis on sixth day of disease.

Feb. 19, 1912, four days before crisis.	Feb. 21, 1912, two days before crisis.	Feb. 23, 1912, one day after crisis.	March 11, 1912, seventeen days after crisis.
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Coagulation time: 8 min., 15 sec.	10 min., 15 sec.	11 min.	6 min., 45 sec.
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Case 17.—480. Severe infection. Died on the seventh day of disease.

Feb. 21, 1912, sixth
day of disease.

Coagulation time:

13 min., 45 sec.

Case 18.—553. Moderately severe infection. Recovered. Pseudocrisis on fifth day of disease. Crisis on eighth day of disease.

March 5, 1912, five days before crisis.	March 7, 1912, day of pseudocrisis.	March, 11, 1912, one day after crisis.	March 26, 1912, sixteen days after crisis.
Coagulation time.			

11 min., 30 sec.	9 min., 30 sec.	11 min., 45 sec.	8 min.
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Case 19.—405. Mild infection. Recovered. Crisis on eighth day of disease.

Dec. 14, 1911, one day before crisis.	Dec. 15, 1911, one day after crisis.	Dec. 21, 1911, seven days after crisis.	Jan. 2, 1912, three weeks after crisis.
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Coagulation time:

6 min., 15 sec.	6 min., 40 sec.	7 min.	8 min., 30 sec.
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Case 20.—401. Severe infection followed by empyema.

Dec. 21, 1911,
fourth day of dis-
ease.

Coagulation time:

12 min., 30 sec.

In seven instances the coagulation time of the blood in approximately normal individuals was determined by the method described. The specimens of blood were obtained from treated cases of syphilis in whom there was no evidence of syphilitic disease of the liver. The average coagulation time of the blood of these individuals was seven minutes and thirty-nine seconds. The shortest time observed was five minutes and fifteen seconds, and the longest time nine minutes. The average coagulation time of patients with pneumonia during the acute stage of the disease was twelve minutes and twenty-seven seconds. The shortest time was six minutes and fifteen seconds, and the longest time eighteen minutes. The average coagulation time at the time of discharge in patients who recovered was seven minutes and thirty-two seconds. The shortest time observed was six minutes and thirty seconds, and the longest, excepting one patient whose blood had last been tested some days before discharge, was nine minutes.

From these examples it is seen that the coagulation time of the blood is definitely prolonged during the acute stage of pneumonia. In one instance, case 20, a very mild infection involving part of one

lobe, the coagulation time during the height of the disease was possibly slightly less than normally. The degree of prolongation of the coagulation time seems to have no definite relationship to the severity of the pneumonic process. The coagulation time tends to become shorter at about the time of the crisis, but probably does not return to normal for some time afterward. At the time of discharge all patients show a coagulation time that is within normal limits, and the average coagulation time at this period corresponds very closely to that observed in approximately normal individuals.

The factor which causes the lengthening of coagulation time in pneumonia was not decisively determined because quantitative estimations of the various elements entering into the coagulation of the blood could not be made. For the following reasons, however, it was believed probable that the long coagulation time was caused by an increase in the antithrombin of the blood. In rough estimations made by centrifugalization, the proportion of the solid element of the clot to the serum has been found to be markedly increased during lobar pneumonia. This striking increase, of course, cannot be accounted for by an increase in the cellular elements and must be due to an increased amount of fibrinogen in the circulating blood. That an increase in the amount of fibrinogen occurs during pneumonia is known and has received experimental confirmation in pneumococcus infections of animals. It is not unlikely that the characteristically large fibrin content of pneumonic exudates depends upon the increased fibrinogen of the blood. Dr. Peabody, in this hospital, has shown that the decrease in calcium content is very slight and is inadequate to explain the changes in coagulation time. Howell⁸ has determined that the formation of fibrin from fibrinogen is not a fermentative process but is a quantitative reaction between thrombin and fibrinogen. Inasmuch as a large firm clot always forms in pneumonic blood, it would seem unlikely that there is a deficiency of thrombin, but that the delay is caused by the presence of some inhibitory substance such as antithrombin. In addition, Whipple⁹ has recently demonstrated by accurate methods, that in an analogous infection the delay in coagulation time is occasioned by an increase in antithrombin.

⁸ Howell, W. H., *Am. Jour. Physiol.*, 1910, xxvi, 453.

⁹ Whipple, G. H., *loc. cit.*

The liver is generally accepted as the principal source of the anti-thrombin of the blood. Meek¹⁰ has recently confirmed the results of other observers in showing that the liver is most important in the regeneration of circulating fibrinogen. The increase in both of these elements of the blood during lobar pneumonia would seem to be due to a common stimulus affecting principally the liver. It is well known that injection into the circulation of animals of certain degradation products of protein induces a prolongation of the coagulation time of the blood. In lobar pneumonia opportunity is afforded for absorption from the lung of large quantities of such substances. Inasmuch as it is shown in the present paper that at the time of the crisis, when probably the greatest quantity of these substances reach the circulation, the coagulation time tends to decrease, it is unlikely that they are the chief factors concerned in the lengthening of coagulation time. The result is more probably produced by some specific effect upon the liver of intoxicating substances originating from the infecting organism.

SUMMARY.

The coagulation time of the blood is generally prolonged during the acute stage of lobar pneumonia, returning to normal during the period of convalescence.

There seems to be a simultaneous increase in the quantity of circulating fibrinogen. The lengthening of the coagulation time is probably due to an increased formation of antithrombin. The source of the increased antithrombin and fibrinogen is probably the liver, and the stimulus to increased production of these two substances is due to the nature of the infecting organism.

¹⁰ Meek, W. J., *Am. Jour. Physiol.*, 1912, xxx, 161.

THE CARBON DIOXIDE CONTENT OF THE BLOOD IN PNEUMONIA.*

By FRANCIS W. PEABODY, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research,
New York.)*

As a result of experimental study and of a limited amount of clinical investigation, it has long been known that fever, or more accurately infection, is very generally associated with a diminution in the carbon dioxide content of the blood. This low carbon dioxide content has been accepted, probably correctly, as an index of acidosis. Apparently the values for carbon dioxide in the blood in fever are not infrequently within the range which is found in the more typical acidosis of diabetes. The object of the present study was to determine more accurately than has yet been done the constancy and extent of the variations in the carbon dioxide content of the blood in pneumonia, the possible relation of such variations to the course or severity of the disease, and more especially the relation of the carbon dioxide content to certain changes in the urine in this disease. Analysis of arterial blood, would, of course, be of much more value than of venous blood, but it is quite impossible to obtain samples of arterial blood from patients except under unusual circumstances. It will be noted, however, that the experimental studies on arterial blood agree well with the clinical studies on venous blood. On account of the recognized bearing of the carbon dioxide content of the blood on acidosis, the nitrogen and ammonia of the urine were quantitated. Determinations of "alkali retention" (Henderson and Adler) were also made, as this method is stated to be an index of acidosis. The relation between chlorine retention and carbon dioxide was studied, and observations were also made on the "total acidity" (Folin), phosphorus, and acetone in the urine.

* Received for publication, August 1, 1912.

The first observations on the gases of the blood in fever were made by Pflüger in 1868 in the course of a series of experiments on dogs. Small operations were performed and the wounds healed with pus formation. Analysis of the blood showed a diminution in the contents of both oxygen and carbon dioxide. Senator¹ made determinations of the gases in the blood of a dog injected with pus. Four hours after injection the temperature had risen to 38.7° C., and the arterial blood showed a fall of 5.6 per cent. in the content of carbon dioxide. The oxygen had remained normal. Reynard's² experiments gave variable results. In one instance fever was produced by the injection of putrifying urine, and a fall in the oxygen and rise in carbon dioxide content of the arterial blood were noted. In two experiments the oxygen was increased and the carbon dioxide diminished. The latter he considered due to the accelerated respiration. In a dog in which septicemia was produced by injecting putrified blood into the femoral vein a fall of both oxygen and carbon dioxide occurred. Geppert³ made a more extensive study of the arterial blood in fever in dogs. The oxygen content he found not to vary from the normal. The carbon dioxide content, on the other hand, dropped considerably, and the diminution appeared to run approximately proportional to the height of the fever. The fall in carbon dioxide even after the onset of a very intense fever was somewhat delayed. Geppert explained the results by supposing that in fever there is a diminution in the alkalinity of the tissues and a subsequent corresponding change in the composition of the blood. He cites as an analogous condition the low carbon dioxide content, associated with a rise of temperature, caused by severe muscular exertion. "As a result of prolonged contractions, the muscle becomes acid, allows acid products to get into the blood, diminishes its alkalinity, and thus explains the fall in carbon dioxide."

Minkowski⁴ made extensive contributions to the subject. Geppert's results had been criticized on the ground that the low carbon dioxide was dependent on rapid respiration. Hence Minkowski first confirmed his observations as to the constancy of the low carbon dioxide in fever, and then showed that the change is quite independent of respiratory rate. Minkowski suggested, as did Geppert, that the essential factor is a change in the composition of the blood,—a diminution of the substances which bind carbon dioxide. Indirect evidence of acid formation in fever is given by the high ammonia output, the frequency with which acetone is found in the urine, and the finding by Minkowski of lactic acid in small amounts in the blood of febrile dogs. Minkowski repeated the experiments of Walter⁵ who found a diminution in the carbon dioxide of the blood of animals fed on mineral acids. Rabbits, which on account of their herbivorous diet have little ammonia at their disposal with which to neutralize acids, showed a greater fall of carbon dioxide than did dogs which were fed on a meat diet. In fever the fall in the carbon dioxide content of the blood is greater in rabbits

¹Senator, H., *Untersuchungen über den fieberhaften Process und seine Behandlung*, Berlin, 1873.

²Reynard, *Recherches expérimentales sur les variations pathologiques des combustions respiratoires*, Paris, 1879.

³Geppert, J., *Ztschr. f. klin. Med.*, 1880-1, ii, 355.

⁴Minkowski, O., *Arch. f. exper. Path. u. Pharmacol.*, 1885, xix, 205.

⁵Walter, F., *Arch. f. exper. Path. u. Pharmacol.*, 1877, vii, 148.

than in dogs. The organism in fever is consuming its own tissues and is thus essentially on a meat diet,—a diet associated with a high ammonia output in which the alkali present is not sufficient to cover the overproduction of acids formed. Another factor suggested for the withdrawal of available alkali for transporting carbon dioxide is the passage of alkali from the serum into the tissue cells, where it is bound by the acid products of decomposition. The retention of sodium in fever was cited. Finally Minkowski found a similar low value for the carbon dioxide in the rise of temperature produced artificially in overheated animals. While probably in part due to increased respiratory rate, other factors, notably metabolic changes and muscular rigor, play an important part, in which again the essential feature is a diminution in the alkalinity of the blood.

That the carbon dioxide content of the blood is diminished during fever had been definitely established by animal experimentation when Kraus^{*} made the first observations on man. His studies embraced a series of fourteen patients with fever of various types. Typhoid and tuberculosis composed the long fevers, and erysipelas, scarlet fever, and pneumonia the short ones. The blood was obtained from the median vein. In general the results were a confirmation of those obtained in animals. While the normal percentage of carbon dioxide in the blood varies, according to him, from 26 to 40.3 per cent. by volume, most of the normal figures run between 31.34 and 35.96 per cent. During fever, however, the carbon dioxide content was between 9.84 and 20.34 per cent. An exception to this was formed by two cases of pneumonia. One of these, on the fifth day of the disease, had a carbon dioxide content of 22.16 per cent., and the other, on the sixth day, a content of 29.2 per cent.,—thus within normal limits. Kraus suggests that the relatively high carbon dioxide content of the blood in the cases of pneumonia may be due to the fact that the sudden lessening of available respiratory surface by the formation of the pneumonic exudate interferes with the excretion of carbon dioxide in the lungs. He found no especial relation between the height of temperature and the diminution of carbon dioxide and suggested that temperature is in general no index of the severity of an infection, but possibly the low carbon dioxide, indicating the more essential metabolic changes in fever, might be some measure of the severity of the disease. In the majority of his cases one analysis of the blood was made but in one instance of pneumonia a second analysis was made a few hours after the crisis, and in three cases of typhoid fever analyses were made at a time when the temperature had been brought down to normal by administering antipyretics. As in none of these was there any marked rise in the carbon dioxide, he concluded that the fall of the fever is not immediately followed by a return of the carbon dioxide to normal. In two cases of erysipelas, however, the second analysis was made after one day without fever, and after the patients had begun to take nourishment. In both the carbon dioxide had risen to normal. Kraus agreed with the former investigators that the diminished carbon dioxide content of the blood in fever is the expression of a lowered alkalinity of the blood. This view, he believed, received support from his method of direct titration of the blood.

^{*} Kraus, F., *Ztschr. f. Heilk.*, 1889, x, 106.

METHODS.

For the purposes of a study in which it was intended that frequent analyses of the blood of patients should be made, and in which very small variations in the results would be of little or no importance, it was desirable to have a method which allowed accurate blood-gas determinations in small amounts of blood, and which was simple enough to be applicable for clinical use. The method described by Barcroft and Haldane⁷ fulfills these requirements. The apparatus and the method of using it have been described so well by its originators that nothing need be added on this point. In general it is as follows: About 1 c.c. of blood, taken under precautions to exclude contact with air, and prevented from coagulating, is put into a small glass bottle which connects with a water manometer. The blood is laked by ammonia solution. By a special device inside the bottle, potassium ferricyanide is added to the laked blood and the oxygen set free. The increase of pressure caused by the gas is measured by the manometer. After resetting the manometer, tartaric acid is added to the blood mixture and the carbon dioxide set free. From the change of pressure shown by the manometer and from the previously determined volume of the bottle and manometer, the volume of gas liberated is calculated. Corrections are made for the solutions used, for temperature and barometric pressure, and for the absorption of carbon dioxide by the fluid mixture in the bottle. The results are then reported in volumes of gas reduced to a pressure of 760 mm. and a temperature of 0° C.

In this work some of the modifications of the original apparatus as devised by Brodie⁸ were used. The "modified blood-gas apparatus" is easier to work with than the original form, and the substitution of a solution of bile salts for water in the manometer greatly simplifies the problem of keeping the manometer absolutely clean. Brodie's method for collecting, measuring, and delivering the blood was tried, but proved rather too complicated for use in connection with patients. Glass syringes provided with metal cases, of the form described by Barcroft and Haldane, were therefore used. In these syringes the blood is mixed with a known amount of 2 per cent. potassium oxalate solution and an accurately measured quantity of blood and oxalate delivered into the gas apparatus. In nearly every instance two separate samples of blood were taken. One disadvantage of this method is that it involves puncturing the skin twice, but with sharp needles the discomfort is slight. Another possible disadvantage is suggested by the experiments of Pflüger,⁹ who obtained somewhat varying results in his sets of blood-gas analyses, unless the samples of blood were taken simultaneously. He states that the difference might be as great if the second sample was taken immediately after the first as if the two samples were taken some hours apart. The blood was taken from one of the veins at the bend of the elbow. On account of the effect of stasis on the blood-gases, it was a general rule that no compression of the arm or obstruction of the circulation should be caused. In the rare cases in which it was not possible to enter the vein

⁷ Barcroft, J., and Haldane, J. S., *Jour. Physiol.*, 1902, xxviii, 232; Barcroft, J., and Morawitz, P., *Deutsch. Arch. f. klin. Med.*, 1908, xciii, 223.

⁸ Brodie, T. G., *Jour. Physiol.*, 1909-10, xxxix, 391.

⁹ Pflüger, E., *Arch. f. Anat. u. Physiol.*, 1868, i, 61.

without some distension of it, compression was made for a very short time, and released as soon as the needle was in the vein. The blood was then withdrawn slowly and only after enough time had elapsed for the circulation to readjust itself.

The application of this method of blood-gas analysis to clinical work presents some difficulties at first, but the results obtained are, for the most part, very satisfactory. The two controls usually checked accurately and almost always gave results within the necessary limits of accuracy of the experiment. Of sixty-five consecutive pairs of analyses of the carbon dioxide in the blood, twenty-four agreed within less than 1 per cent. by volume, forty-three within 2 per cent., and fifty-one within 3 per cent.

According to Loewy,¹⁰ the arterial blood of man contains between 39 and 43 per cent. of carbon dioxide at a tension of 30 to 40 mm. of mercury, while venous blood contains from 43 to 50 per cent. of carbon dioxide at a tension of 40 to 50 mm. of mercury. The figures obtained in this investigation for the normal carbon dioxide content of the venous blood are somewhat higher than those given by Loewy, but they are very constant, both in healthy individuals and in the patients after their metabolic processes had returned to normal. In general, the normal carbon dioxide of the venous blood varied between 54 and 58 per cent.

The methods used in the analysis of the urine were as follows: "Alkali retention" was determined according to the method of Henderson and Adler.¹¹ A definite quantity of urine, under standard conditions, is titrated with one-tenth normal sodium hydrate solution, using neutral red as an indicator, until it is of the same reaction as a standard solution which is made up to the reaction of blood. This titration is supposed to give an index of the amount of acid excreted by the kidney, and corresponds to the amount of base saved to the body. To this figure is added the ammonia excretion, expressed also in terms of one tenth normal solution. The sum of the two measures "the effective work of the kidney in saving basic substances for the further neutralization of acid and transport of carbonic acid." The other determinations were done by the usual methods,—total nitrogen by Kjeldahl's, ammonia by Folin's,¹² chlorine by the Volhard method,¹³ phosphorus by titration with uranium nitrate solution,¹⁴ acetone by Lieben's test, and "total acidity" by the method of Folin.¹⁵

THE CARBON DIOXIDE CONTENT OF THE VENOUS BLOOD.

Observations on the carbon dioxide content of the venous blood were made on twenty-six cases of pneumonia. Of these, eleven

¹⁰ Loewy, A., in von Koranyi, A., and Richter, P. F., *Physikalische Chemie und Medizin*, Leipzig, 1907, i, 255.

¹¹ Henderson and Adler, *Jour. Biol. Chem.*, 1909, vi, p. xxxviii; Adler, H. M., and Blake, G., *Arch. Int. Med.*, 1911, vii, 479.

¹² Folin, O., *Am. Jour. Physiol.*, 1905, xiii, 45.

¹³ Harvey, S. C., *Arch. Int. Med.*, 1910, vi, 12.

¹⁴ Neubauer-Huppert, *Analyse des Harns*, 11th edition, Wiesbaden, 1910, i, 138.

¹⁵ Folin, O., *loc. cit.*

were fatal cases. In all, ninety-one analyses were made. With the exception of two cases, which will be discussed later, the carbon dioxide was found to be regularly diminished during the febrile period. In most cases the carbon dioxide content ran from 40 to 50 per cent. in the acute stage of the disease, but not infrequently it was from 50 to 53 per cent., or only very slightly below normal. The lowest observation made was 29.01 per cent.

No definite relationship could be made out between the carbon dioxide content of the blood and either temperature, pulse, or respiration, except that the lowest values for carbon dioxide were apt to be found associated with very rapid respiration. Nor was there any clear-cut relation between the carbon dioxide and the extent of lung involved or the severity of the disease. Four cases, however, in which observations were made within two hours of death, are of considerable interest, as they show that the tendency is for the carbon dioxide content to be very low in the terminal stage of the disease. When, however, circulatory disturbances or local processes in the lungs interfere with the respiratory exchange of gases, the carbon dioxide may be relatively higher. In case XIX the chest was free from râles two hours before death, and the oxygen content of the blood was normal. Gaseous exchange in the lungs was thus apparently unaffected. The carbon dioxide content was 29.01 per cent., the lowest value found in the whole series. Case XVI was very cyanotic and had a general edema of the lungs one hour before death. An exceedingly low oxygen content of the venous blood suggests that oxygen was being imperfectly taken up in the pulmonary capillaries, and makes it probable that the excretion of carbon dioxide was similarly interfered with. In spite of this, the carbon dioxide was only 35.53 per cent. The same conditions, pulmonary edema and low oxygen content, prevailed in case XII two hours before death, and here again the carbon dioxide value was one which would be considered low, even if there were no hindrance to the excretion of carbon dioxide in the lungs. Similarly, case XXIV had, on clinical examination thirty minutes before death, marked cyanosis and many coarse tracheal râles. At this time the oxygen content of the venous blood was only 2.07 per cent., and the carbon dioxide content was

40.46 per cent. Thus the three last cases show a carbon dioxide content which is absolutely low and may be considered to be relatively very low. Beyond this, there is little association between the severity of the disease and the carbon dioxide of the blood, except that the mild cases with a short course usually showed the least marked deviations from the normal.

The relation of the carbon dioxide content of the blood to the course of the disease is somewhat variable. Unfortunately, no observations were made on the first or second days. Several cases, however, notably Nos. III, VIII, IX, and XXI, show that the carbon dioxide is, in general, nearer normal in the first days of the disease, and then gradually sinks, so that the minimum is at about the height of the disease or at the time of the crisis. After the crisis the carbon dioxide usually begins to show a rise towards normal, but occasionally very low, and even the lowest figures are found some days after the patient has become afebrile. Thus case XXI had a carbon dioxide content of 43.08 per cent. four days after the crisis, and case III one of 45.23 per cent. on the fifth day of normal temperature. Both of these patients had been taking a good amount of nourishment for several days, so that the low carbon dioxide can scarcely be referred to a starvation acidosis. These cases bring out the fact that the low carbon dioxide content of the blood may persist for a considerably longer period after the fall of temperature than was noticed by Kraus, and they show definitely that the changes in the carbon dioxide bear no direct relation to temperature itself, but are rather in line with the other metabolic changes which are associated with infection and which are sometimes more marked after the fall of temperature than during the febrile period itself. Chief among the evidences of these changes, as shown in the urine, are the high nitrogen and ammonia excretion ("epicritical excretion") and the low chloride excretion.

THE RELATION OF THE CARBON DIOXIDE CONTENT OF THE BLOOD TO THE EXCRETION OF AMMONIA AND CHLORINE IN THE URINE.

The high excretion of ammonia and the low excretion of sodium chloride are two of the most characteristic features of the urine during fever. The diminution of the carbon dioxide in the blood

15	98.0	72	20	—	4,300	17.01	0.94	5.35	21.99	4.34	040.1	011.2	28.9	533.8	070.5	1224.3
6	90.2	56	22	—	2,590	23.81	0.56	2.37	16.63	5.00	885.9	704.2	181.7	764.0	398.5	1163.5
7	98.2	48	20	—	1,310	14.46	0.46	3.15	9.53	3.55	481.0	500.0	—	411.3	327.3	738.6
	98.7	58	24	—												
	97.4	52	22	—												
	97.4	52	22	—												

is apparently as constant an accompaniment of fever. It would be of some interest, then, to see in how far these three changes run parallel to one another. This is especially so since Hamburger¹⁶ has attempted to explain the chloride retention on the basis of a febrile acidosis.

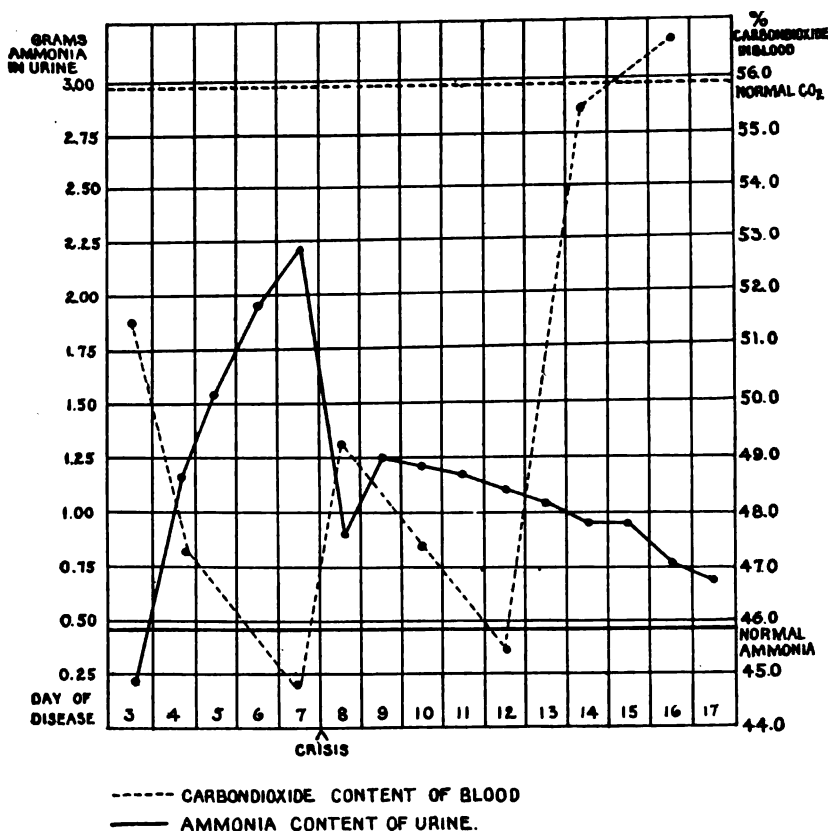
In general, of course, the excretion of ammonia and the excretion of chlorides are in inverse ratio during fever. When the ammonia excretion is high, the chlorine excretion is low. After the fever is over, the ammonia excretion falls to normal and the chlorine excretion rises to normal or above. The curves of excretion of the two substances, however, do not run strictly parallel to one another. The ammonia runs fairly well parallel to the total nitrogen excretion,—so much so that the percentage of ammonia nitrogen is usually not far from normal. Occasionally it is somewhat high during the febrile period. That the changes in chloride and in ammonia excretion do not always run strictly parallel is shown by cases III and VIII (table I), in which the chlorides began to be excreted two days after the crisis, and the ammonia only reached a normal level four or five days later. The same tendency for the ammonia metabolism to readjust itself more slowly than the chloride metabolism is seen, but less strikingly, in other cases.

Kraus¹⁷ has already shown that the carbon dioxide content of the venous blood does not necessarily rise to normal immediately after the fall of temperature. His examinations were made after one or two days of apyrexia. Moreover, we have already seen that in this series of cases the carbon dioxide may persist at a low level for a considerably longer period after the fever has gone. In case III (text-figure 1) it was as low as 45 per cent. five days after the crisis. Two days later it had risen to a normal value. The return of the carbon dioxide to normal was in this instance at just about the period at which the ammonia excretion in the urine was becoming normal. Retention of chlorine had ceased some days before. Case IV shows similarly a lack of relation between the carbon dioxide content and the chlorine excretion. The carbon dioxide was 47.1

¹⁶ Hamburger, H. J., *Osmotischer Druck und Ionenlehre in ihrer Bedeutung für die Physiologie und die Pathologie des Blutes*, Berlin, 1912.

¹⁷ Kraus, F., *loc. cit.*

per cent. on the eighth day after the crisis, nearly a week after chlorine retention had ceased. The ammonia excretion was irregular but only became constantly normal after this time. In case VIII the carbon dioxide was still low at the time when the chlorine was just beginning to come out.



TEXT-FIG. I. The relation between the carbon dioxide content of the blood and the ammonia content of the urine in case III.

It is thus only in the few cases in which there is a very definite difference in the time at which the metabolism of ammonia and that of chlorine readjust themselves, that the relation of the carbon dioxide to each of them can be studied. The evidence, however, goes to show that the carbon dioxide in the blood follows much more

closely the curve of ammonia excretion than it does that of chlorine. In view of the known relation between ammonia excretion and carbon dioxide in typical acidosis (experimental acidosis, diabetic acidosis), this is, of course, what one would have expected *a priori*. Hopkins and Davis¹⁸ have brought out the interrelation of carbon dioxide and ammonia from a different point of view by showing experimentally that asphyxiation, by increasing the carbon dioxide content, causes a decrease in the ammonia content of the blood. Case XVII (table II) shows this relationship even more definitely. At a time when the carbon dioxide was 43.64 per cent. in the blood, the patient was given sodium bicarbonate in such amounts that the urine was made alkaline. In the forty-eight hours preceding his death, six specimens of urine were obtained by catheter. Examinations of the blood were made to correspond to each specimen of urine. It will be seen that the administration of alkali caused an increase in the carbonates of the blood (the carbon dioxide pressure was higher than could be read on the manometers), a change in the reaction of the urine, and a great fall in the ammonia excretion. When the sodium bicarbonate was discontinued, the carbon dioxide in the blood fell and the ammonia in the urine rose nearly to its former value. The chlorine excretion showed no rise with the administration of alkali, but rather a gradual falling off until death. The acetone reaction was very strong in all specimens and showed no relation to the carbon dioxide in the blood, the ammonia or reaction of the urine. In the alkaline specimen of urine the amount of albumen was much less than in the acid specimens.¹⁹

Case XXVI (table III, text-figure 2) shows the same condition. On the third day of the disease, control observations were made on the blood and urine. The carbon dioxide in the blood was moderately low, and the urinary ammonia only slightly high, as is typical of the first days of the infection. The patient was receiving ten grams of sodium chloride by mouth daily in addition to the salt in his food. Of this he was retaining about two-thirds. For the following two and a half days, sodium bicarbonate was given by

¹⁸ Hopkins, R., and Davis, W., *Jour. Biol. Chem.*, 1911, x, 407.

¹⁹ von Hösslin, R., *Deutsch. Arch. f. klin. Med.*, 1912, cv, 147.

TABLE II.
Case 17.

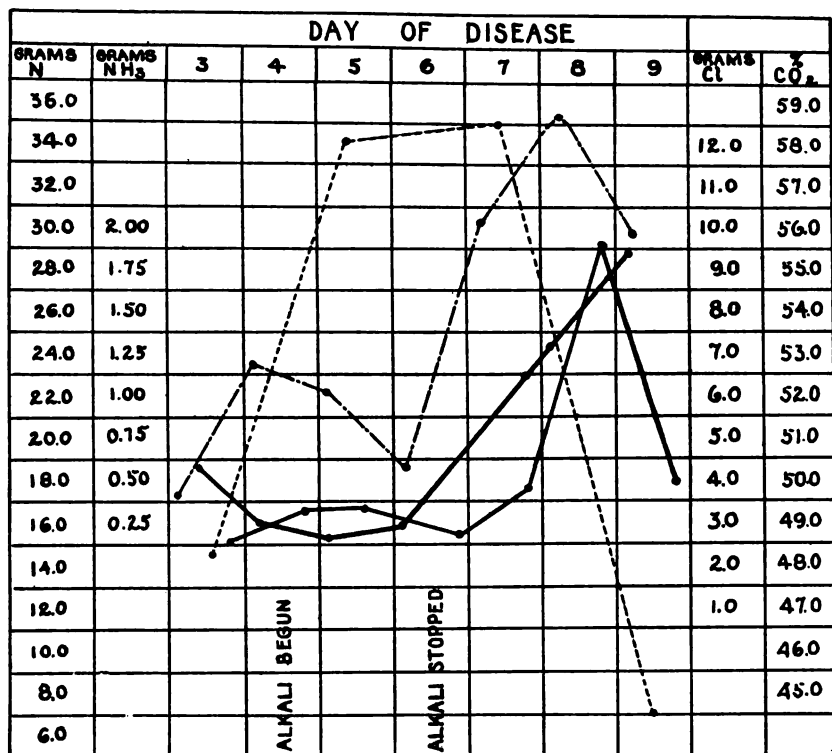
Num- ber of speci- men.	Time.	Hours.	Amount in c.c.	Reaction.	Specific gravity.	Albu- men.	Nitro- gen.	Am- monia.	Percent- age of am- monia.	Chlo- rine.	Percent- age of chlo- rine.	Ace- tone.	Time.	Carbon dioxide.
I	9:00 A. M. to 6:00 P. M.	9.0	600	Acid	1.021	++	10.36	0.280	2.7	0.42	0.070	++	10:00 A. M.	43.64
II	6:00 P. M. to 5:00 A. M.	11.0	770	Alkaline	1.020	++	7.67	0.033	0.4	0.14	0.018	++	4:30 A. M.	74 +
III	5:00 A. M. to 10:30 A. M.	5.5	640	Alkaline	1.015	++	4.97	0.005	0.1	0.16	0.025	++	9:30 A. M.	74 +
IV	10:30 A. M. to 4:30 P. M.	6.0	490	Alkaline	1.017	++	4.65	0.022	0.4	0.12	0.025	++	2:30 P. M.	62.95
V	4:30 P. M. to 10:00 P. M.	5.5	340	Slightly acid	1.016	++	4.18	0.084	2.0	0.06	0.018	++	8:45 P. M.	55.27
VI	10:00 P. M. to 9:00 A. M.	11.0	355	Acid	1.017	++	4.57	0.271	6.1	0.00	0.000	++	10:00 A. M. 1:00 P. M.	64.62 62.10

TABLE III.
Case 26.

* Day of disease.	Total amount.	Reaction.	Nitro- gen.	Am- monia.	Percent- age of am- monia.	Chlo- rine.	Albumen.	Carbon dioxide.	Remarks.
3	1,320	Acid	18.04	0.70	3.8	3.19	Very slight	48.92	Sodium bicarbonate 5 gm. every two hours, 9:00 A. M. Sodium bicarbonate 5 gm. every four hours, 8:30 P. M. Sodium bicarbonate discontinued, 11:30 P. M.
4	2,390	Alkaline	24.65	0.37	1.5	3.74	Slight	—	
5	2,555	Alkaline	22.92	0.34	1.4	3.93	Slight	58.51	
6	2,250	Alkaline	19.79	0.42	2.1	3.33	Very slight	—	
7	2,580	Alkaline	30.70	1.29	4.2	4.38	Very slight	59.15	
8	3,740	Neutral	36.50	1.45	3.9	10.20	Very slight	—	
9	2,320	Acid	30.07	1.96	6.5	4.64	Very slight	45.00	

The patient received 10 gm. of sodium chloride daily.

mouth in rather large doses. The urine became alkaline, the carbon dioxide in the blood rose, the ammonia fell to a low absolute, and a very low relative figure. In spite of a greatly increased diuresis, there was very little change in the chlorine excretion except



----- TOTAL NITROGEN IN URINE ——— AMMONIA IN URINE
 ——— CHLORINE IN URINE ----- CARBONDIOXIDE IN BLOOD

TEXT-FIG. 2. The effect of feeding sodium bicarbonate upon the carbon dioxide content of the blood and the ammonia content of the urine in case XXVI.

on the eighth day, after the administration of sodium bicarbonate had been stopped, and when the urine had become neutral once more. On this day, with much the largest total amount of urine, the chlorine rose to over ten grams. The ninth day served as a further

control. The urine was again acid, the ammonia was absolutely very high and relatively slightly high, the chlorine excretion was a little higher than before and the carbon dioxide content of the blood was once more low. In none of the specimens was there more than a faint cloud of albumen. Thus the question of chloride retention was not complicated by acute nephritis.

In these two cases, then, with great variations in the carbon dioxide content of the blood, exactly corresponding changes were found in the ammonia output in the urine. No similar interrelation was to be found between the carbon dioxide and the excretion of chlorine.

So far, then, the evidence points against the theory that the retention of sodium chloride in fever depends on the acidosis. Further evidence against such a theory is given by the conditions found in more typical varieties of acidosis. In diabetic acidosis and in experimental poisoning by mineral acids, there is not a retention of bases as there is in pneumonia, but a marked increase in the excretion of bases. The conditions, then, with regard to the excretion of bases are diametrically opposed to one another.

THE RELATION OF THE CARBON DIOXIDE CONTENT OF THE BLOOD
TO THE PHOSPHATES, THE "TOTAL ACIDITY," AND TO THE
"ALKALI RETENTION" OF THE URINE.

Phosphates.—In agreement with the usual results in fever, the excretion of phosphates was rather irregular. In general, however, the urine contained larger amounts after the temperature had fallen than in the febrile period. There is apparently no especial relation between the carbon dioxide content of the blood and the excretion of phosphates. The latter frequently rises after the crisis, quite irrespective of any change in the carbon dioxide.

"Total Acidity."—This term is applied by Folin²⁰ to the value which is obtained by titrating the urine with sodium hydrate after the addition of neutral potassium oxalate, phenolphthalein being used as an indicator. It is expressed in cubic centimeters of decinormal sodium hydrate solution. According to Folin,²¹ "the phosphates in

²⁰ Folin, O., *loc. cit.*

²¹ Folin, O., *loc. cit.*, p. 66

clear acid urine are all monobasic, and the acidity of such urines is ordinarily greater than the acidity of all the phosphates, the excess being due to free organic acids." The acidimetric value of the free organic acids is thus obtained by subtracting the acidimetric value of the phosphates from the "total acidity." "If the acidity calculated from the total phosphates is greater than the titrated acidity, then there are practically no free organic acids present, and the titrated acidity represents the amount of phosphates present in the diacid form."²² Magnus-Alsleben²³ used this method in the examination of the urine of children with scarlet fever, and found a considerable increase in the organic acids during the febrile period. After the temperature became normal, the phosphate acidity usually exceeded the "total acidity." The children were on a milk and vegetable diet. He believes that the method gives a simple means of obtaining a numerical value for the abnormal excretion of acids during fever.

Pick²⁴ has described a similar condition in the urine of pneumonia. In forty-two out of fifty-four cases there was a change in the reaction of the strongly acid urine, so that in thirty-six to forty-eight hours after the crisis it became less acid, amphoteric, or alkaline to litmus. This was associated with a drop in the proportion of monobasic phosphates as calculated by him. A simultaneous rise in the excretion of dibasic phosphates occurred, and this he considered to be associated with an increased excretion of bases which had presumably been absorbed from the pulmonary exudate. The importance of the rôle of the exudate is, however, somewhat lessened by the fact that the same condition may be found in scarlet fever.

In the present series of cases the results of Magnus-Alsleben are to a considerable extent confirmed. Values representing a rather high excretion of organic acids were practically constant during the febrile period. In several cases (Nos. II, III, and VII) after the fall of temperature, the urine contained no organic acids and the acidimetric value of the phosphates exceeded the "total acidity."

²² Folin, O., *loc. cit.*, p. 55.

²³ Magnus-Alsleben, E., *Ztschr. f. klin. Med.*, 1911, lxxiii, 428.

²⁴ Pick, F., *Deutsch. Arch. f. klin. Med.*, 1900, lxxviii, 13.

In other cases, however (Nos. V, VI, and VIII), the excretion of organic acids was apparently as great after the fall of temperature as during the fever. No parallelism could be found between either the "total acidity" or the excretion of organic acids, as determined by this method, and the carbon dioxide content of the blood.

"Alkali Retention."—The method of determining "alkali retention" has been discussed above. The result is supposed to give a more accurate index of the degree of acidosis present than does the determination of ammonia alone. In the present cases of pneumonia the values which represent "retained bases," and which were obtained directly by titration of the urine to the reaction of blood, were variable and not particularly suggestive. The results obtained for "alkali retention" were, in general, somewhat higher during the fever than after it, but the variation is chiefly due to the ammonia excretion. The relation of the "alkali retention" to the carbon dioxide content of the blood is less definite than is that of the ammonia alone.

ATYPICAL CASES.

It has been stated that two cases (Nos. II and V) were atypical in that the carbon dioxide content of the blood was normal or above normal during the febrile period. One of these (No. V) was a case of influenzal pneumonia with involvement of the right lower lobe, and the other a case of pneumococcus pneumonia with solidification of the left lower lobe. The first observation on the carbon dioxide of each case was slightly below normal, but all subsequent observations were normal or above normal. Both were fairly severe cases. The urinary analyses gave results which do not differ from those in ordinary cases of pneumonia,—a moderately high ammonia output, and a marked or moderate retention of chlorides. The diet was essentially the same as in the other cases, and contained nothing which would raise the carbonates in the blood. The cause of this unusually high carbon dioxide is suggested by the analyses of the oxygen content of the blood made at the same time. In uncomplicated cases of pneumonia the diminished respiratory surface is compensated for, and the amount of oxygen in the venous blood re-

mains fairly well within the normal limits of 11 to 12 per cent. In no other uncomplicated cases were oxygen values found which were nearly as low as in these two cases. Both cases, moreover, showed clinically a cyanosis which was more extreme than was seen in any of the other cases. Cyanosis, a low content of oxygen, and a high content of carbon dioxide, suggest, of course, an interference with the respiratory gas exchange, an incomplete absorption of oxygen and an incomplete excretion of carbon dioxide. In case II, at the first observation, the oxygen content of the blood was normal and the carbon dioxide was, as one would expect, low. On the following days, when the oxygen was low, the carbon dioxide had risen markedly, and only when the oxygen rose again to normal did the carbon dioxide fall. Practically the same association of low oxygen and high carbon dioxide is seen in case V.

Such an imperfect aeration of the blood might conceivably depend on an incomplete compensation for the diminution of respiratory surface caused by the pneumonic exudate, although it is scarcely possible that the throwing out of one lobe should produce so profound a change. According to Loewy and von Schrötter,²⁵ the occlusion of the main bronchus to one lung, without change in rate of respiration or circulation, would only lower the oxygen content of the venous blood to 8 per cent. Moreover, in case V the low oxygen and high carbon dioxide persisted for some time after the chest was perfectly clear. That the low oxygen was due to an inability of the blood to take up oxygen normally was disproved in case V at least by finding, on three occasions, that the combining power of the blood for oxygen was normal. That the time of the reaction might be slowed, however, cannot be excluded. In neither case was there any reason to believe that improper aeration depended on too shallow respiration. In case V the pulse rate was not rapid, but in case II it was about what one would expect with the amount of fever present. An unusual slowing of the rate of circulation would increase the amount of oxygen given off in the capillaries, and also the amount of carbon dioxide taken up, and thus account for the changes found in the venous blood. Wolff²⁶ has

²⁵ Loewy, H., and von Schrötter, H., *Ztschr. f. exper. Path. u. Therap.*, 1905, i, 197.

²⁶ Wolff, E., *Arch. f. exper. Path. u. Pharmakol.*, 1885, xix, 265.

shown that in rabbits the circulation in fever is actually slower than normally. More recently Hewlett²⁷ has concluded from studies on man that "the flow is exceptionally slow when the temperature rises during fever and is moderately accelerated when the temperature falls during fever." If this be so, then in the great majority of cases of pneumonia, the slowing of the circulation, as well as the diminished respiratory surface, must be compensated for by changes in respiration. From the facts at hand, however, it would be impossible to determine whether the unusual condition of the blood in these cases is due to a slowing of the circulation or to an interference with respiratory exchange of gases due, possibly, to some local change in the lungs, as of the capillary walls or the alveolar cells, or to the effect of a change in the chemical constitution of blood cells or of serum. Kraus found that the carbon dioxide content of the blood was considerably higher in pneumonia than in other acute fevers, and he explained this by the pulmonary involvement which complicates the conditions in pneumonia. According to him, the local conditions interfere with the ready excretion of carbon dioxide. In the presence of a normal oxygen content, as is found in most cases, this would be difficult to prove, but the explanation seems worth considering in these atypical cases.

CONCLUSIONS.

A diminution in the carbon dioxide content of the blood is a constant feature in pneumonia. Occasional cases, however, may fail to show low carbon dioxide.

The carbon dioxide in the blood bears little definite relation to the severity of the disease, except that it tends to be lowest in severe cases and in the terminal stages of the disease. There is less deviation from the normal in short or mild cases.

The diminution in the carbon dioxide in the blood bears no immediate relation to temperature, as it may persist for some days after the patient is afebrile. The diminution in carbon dioxide corresponds to the other evidences of metabolic changes in infection and, like them, may be even greater after than during the febrile period.

²⁷ Hewlett, A. W., *Heart*, 1910-11, ii, 230.

The changes in the carbon dioxide content of the blood run parallel to the output of ammonia in the urine. The carbon dioxide appears to bear no relation to chlorine excretion.

In two unusual cases the carbon dioxide content of the blood was normal or above normal. This was associated with a very low oxygen content of the venous blood.

A DESCRIPTION OF A CASE OF COMPLETE HEART-BLOCK, INCLUDING THE POST-MORTEM EXAMINATION.

By ALFRED E. COHN,

(*From the Hospital of The Rockefeller Institute for Medical Research,
New York*)

AND

THOMAS LEWIS.*

(*From the Cardiographic Department, University College Hospital Medical
School, London.*)

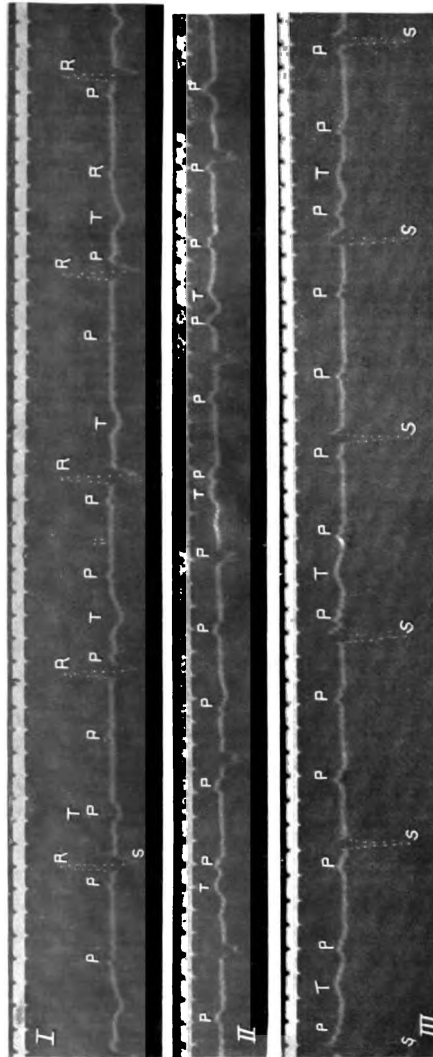
A minister, aged 77, who was a patient of Dr. C. E. McDade, was brought to University College Hospital Medical School in March, 1911, for electro-cardiographic examination, through the kindness of Dr. H. Batty Shaw.

He had been under Dr. McDade's care for a number of years, and his condition was reported in the *Lancet*, 1906, ii, page 653.

The patient was an extremely hard working man who had had excellent health until 1904, when he was 70 years of age.† In July of that year and in February, 1905, he had attacks of vertigo and flatulence. In June, 1905, the pulse rate was noticed to be 40; the beats were strong and regular. He complained of easy exhaustion, especially after exertion. There was then no vertigo, and breathing was free and the urine normal. The heart sounds were clear. On June the 23rd, the pulse beat regularly at 78. From July the 7th to 14th, the pulse beat regularly at 40 and he was performing his usual arduous work without fatigue. On August the 13th, the pulse was regular at 63, and on November the 13th, regular at 38. On December the 15th he seemed to be failing in strength. The pulse was

* Working under the tenure of a Beit Memorial Fellowship.

† For these notes we are indebted to Dr. McDade and to his *Lancet* report.



32 and sometimes irregular. He took to bed on the 19th and the pulse became regular again, varying in rate from 28 to 32. At this time rapid oscillations of the jugular veins were seen. On January the 25th, 1906, a trace of albumen was found in the urine; the pulse remained slow. On February 5 the rate was 63, the beats being regular. For the next four weeks it varied between 48 and 80. By March the 15th, 1906, the pulse rate was normal and active work was resumed in May.

The patient's condition remained satisfactory till October, 1907, when the pulse again fell to 40 and continued at or about this rate until May, 1908. Most of this period was spent in bed or indoors. Albuminurea was present at this time. There had been no further attacks of vertigo.

From May to November, 1908, the pulse was normal and the albuminurea vanished, work being resumed. In November, 1908, the pulse rate fell to 37 and continued at or about the rate of 34 until death occurred in April, 1911. The albuminurea also reappeared and was maintained; moreover the feet and legs became swollen and this condition persisted. At this time the systolic blood pressure was measured at 220 mm. Hg.

On October the 22nd, 1910, a cough developed and strained him a good deal; and a few days later he had a fit of unconsciousness accompanied by lividity.

From January to March, 1911, feebleness increased. Oedema of the ankles and albuminurea were continuously present.

On March the 24th he came to University College Hospital Medical School. The curves, which are reproduced (Fig. 1), demonstrated complete heart-block; the auricular rate was 88 and the ventricular 34. The shape of the ventricular complexes in the three leads was such as is said to indicate hypertrophy of the left ventricle. At this time there was a good deal of breathlessness. With the exception of a systolic apical murmur, the sounds were normal.

In the morning of April the 5th, 1911, he complained of "stupid feelings" in the head; in the afternoon he became gradually comatose and died.

The post-mortem, which was performed by Dr. McDade, was limited to the heart and aorta. The latter were very atheromatous.

The heart was fixed in Müller-Formol, as described in previous communications.

MACROSCOPIC ANATOMY OF THE HEART.

The ventricle measures 13 cm. anteriorly from the *A-V* groove to the apex and 11.5 cm. posteriorly. Its weight is 732 grammes.

On the surface of the heart, more especially over the right ventricle, there is a layer of more than the normal amount of fatty tissue. The pericardium in numerous places is thicker and whiter than normal. The right auricle is dilated a little and the endocardium is opaque. The *tænia terminalis* is not hypertrophied. The tricuspid valve admits four fingers easily; its edges are thickened. The cavity of the right ventricle is not enlarged. The muscle breaks easily under the finger. There are no antemortem dots in any of the chambers. Its wall measures 6 mm. at the base, and 6 mm. at the *conus arteriosus*. The *corpora Arantii* of the pulmonary valve are slightly thickened. The left auricle is considerably dilated; its endocardium is white, thick and smooth. The mitral valve admits three fingers in line. The edges are thick like those of the tricuspid valve. The aortic flap of the mitral valve shows advanced atheroma. The cavity of the left ventricle is slightly dilated, the dilatation being most prominent below the aorta in the outflow tract. The wall at the venous base measures 15 mm., at the level of the papillary muscles it measures 16 mm., and at the apex 10 mm. The *trabeculæ* are flattened. The endocardium over them is smooth and thin, but that covering the membranous septum and the adjoining areas is very thick and firm. The *corpora Arantii* of the aortic cusps are thick and at their attached margins their density is cartilaginous; they were slightly incompetent in the fresh state. The anterior sinus of Valsalva, the line of attachment of the aortic cusp, is calcareous. The other sinuses of Valsalva present an advanced athero-sclerosis. Advanced athero-sclerosis is also found in the coronary arteries. The descending branch of the left coronary artery shows a calcareous plaque near its origin, and also farther along in the course of the vessel. Similar changes are found in the right coronary vessel. The lesions do not constrict the

lumen of these vessels. There is no lesion at or near the septum membranaceum to indicate that the *A-V* system has been compromised. The foramen ovale is closed. Tissues were excised for microscopic examination: (1) Two pieces of the wall of the left ventricle, (2) a block of tissue at the junction of the superior vena cava and the right auricular appendix, containing the sino-auricular node, (3) a block of tissue from the interventricular septum, containing the *A-V* node, main stem and the upper portions of the right and left branches of the *A-V* bundle.

MICROSCOPIC EXAMINATION.

Pieces of tissue from the left ventricle show many connective tissue scars, in which the muscle fibres are either atrophic or destroyed.

Sino-Auricular Node.—This node is found in 2,140 sections. Of these 1,000 were cut 8 micra and 1,140 were 9 micra thick. The total length of this node is therefore 18,260 micra or 18.26 mm. It lies 2 mm. from the pericardium. At the point where it is first seen, the superior vena cava has already joined the right auricular appendix, so that it begins below the level of the angle of this junction. Actually the superior vena cava still shows its complete lumen and it has not yet widened out into the cavity of the right auricle. The node therefore does not lie in the wall of the superior vena cava. In the upper portions it consists of little more than loose-meshed connective tissue which contains about a half-dozen thin and somewhat elongated muscle fibres. It is completely surrounded by fatty tissue. Large nerve trunks and a small vessel lie in its immediate neighbourhood. At a slightly lower level the node lies 1.5 mm. from the pericardium, measures 2.5 mm. from side to side, and extends for 1.5 mm. into the substance of the auricular wall. Its shape is roughly triangular. The pericardium over the node is thick. The size of the artery to the node has increased; the adventitia is hypertrophied. The various structures which form the node differ from the description usually given, because there is very little interlacement. Its muscle fibres sometimes contain vacuoles. At a still lower level, the vessels to the node, both artery

and vein, are prominent and occupy the greater portion of its structure. Its position, size and shape remain unchanged. At its upper extremity it is almost completely surrounded by fatty tissue so that it has an island-like appearance. Farther down the shape becomes more definitely triangular; it lies 2.5 mm. from the pericardium and measures 2.5 mm. from side to side, and is 3 mm. deep. The apex of this roughly triangular structure communicates freely with the auricular muscle. At a still lower level, the node is flatter and longer, the long diameter being parallel to the pericardium; it is 1 mm. deep and lies only 1.5 mm. from the pericardium. The tail, the lowest level of the node, is about 2 mm. from the pericardium and measures 1.5 mm. by 1.5 mm.; its shape is triangular as in the upper levels.

To sum up, the node is almost 2 cm. in length in the fixed state. It lies close under the pericardium through its whole extent. It is first seen at the angle where the superior vena cava joins the right auricular appendix, but it does not extend so high as to lie in the wall of the superior vena cava. Its shape above is first roughly triangular, but at lower levels it is flattened and elongated, while the tail is again triangular. The node is peculiar in that the amount of contained muscle tissue is small relative to the size of the entire structure and that the fibres interlace very little. There is a good deal of connective tissue, but the amount is within the limits of normal variation. It is also peculiar because it is isolated in so large a part of its length by fatty tissue. A feature of the artery to the node which is commented on by Oppenheimer is shown in this case. It consists of the presence of small bundles of muscle, arranged in longitudinal fashion, outside the inner circular layer of the media. The arrangement is similar to that seen in the central veins of the suprarenal gland.

The Auricular and Ventricular Septa.—The cross section of the interauricular septum, the plane of the section being at right angles to the long axis of the heart, shows an unusual preponderance of fatty and connective tissues, over the muscle, which usually form its substance. This change in relation is more apparent in the dorsal portions. In the interventricular septum numerous scars formed

of dense connective tissue, some of which have undergone hyaline degeneration, are seen at all levels. In these scars, the muscle tissue is either atrophic or destroyed. The muscle fibers which form the interauricular septum vary a great deal in their diameter. Their nuclei are different in size and the perinuclear space is wide and free from muscle fibrils, so that the general appearance of the fibres suggests that of Purkinje cells. These fibres are neither grouped nor arranged in a specific order, so that it is impossible to attribute a separate function to them, as has been done. They represent a variety of muscle atrophy. The vessels of the septum, more especially the posterior coronary artery, show an advanced grade of athero-sclerosis. Advanced sclerosis is also seen at the root of the aorta, more especially in the sinus of Valsalva of the left posterior flap. In the corpus Arantii of one of the aortic cusps, cholestrin crystal spaces are seen. There is no evidence of recent inflammation.

The Auriculo-Ventricular Node lies, as usual, to the right of the central fibrous body, it is very small, and is compressed laterally. It communicates with the auricular muscle (the auriculo-nodal junction) by means of a well developed strand of muscle to the left, but by a much attenuated strand to the right. In the upper levels the relative size of these two strands is the reverse of the usual condition, but lower down it is normal. Between the two strands a mass of fatty tissue is found, continuous with the fatty tissue described in the interauricular septum. The auriculo-nodal junction and the auriculo-ventricular node itself are smaller than normal. The node is recognized mainly from its position. The fibres do not interlace in the usual fashion, they are coarser and do not show the normal number of nuclei. The artery commonly found in relation to the node is wanting. Near the node, however, a vessel is found, the structure of which, in contrast to that of the other arteries at this level, is normal. Thus, the continuity of the muscle structure from the auricle to the main stem of the *A-V* bundle is maintained by a node which in appearance differs little from the auricular muscle. At the transition from the node to the *main stem* of the auriculo-ventricular bundle, there are masses of dense connective tissue. In addition to these there is much loose-meshed connective tissue sepa-

rating the individual muscle fibres. A number of blood sinuses are also found. They consist of a single layer of endothelial cells surrounded by walls of dense connective tissue, free from muscle. They occupy a considerable portion of the bundle, while the number of muscle fibres is very much reduced. After a short course in the membranous septum, the main stem lies under the endocardium of the left ventricle. A portion of the fibers now passes dorsally and lies between the endocardium of the left ventricle and the lowermost portion of the central fibrous body. This branch, thin and small at its origin, spreads out later under the endocardium and comprises the left branch. At a lower level the mass of tissue forming it becomes greater. Compared with the left branch, the right branch is large, although the number of muscle fibres contained in it is relatively small. The pathological change, consisting of the old connective tissue and blood sinuses described in the main stem, is continued in the right branch to the lowest level examined. Here few muscle fibres are seen in it and the entire structure is very small. At this level the left branch is well developed and presents no abnormality. The smooth muscle fibres, which are described by Nagayo and which are found in the endocardium of the left ventricle and between it and the *A-V* system which lies in the outflow tract, are well developed. The fibres of the left branch show vacuoles. The difference in colour between the muscle of the conducting system and the myocardium is well seen in this case.

To sum up, in the conducting system of this heart the auricular nodal junction is smaller than usual. The auriculo-ventricular node is flattened and small and it is not characteristic either in the arrangement of its muscle or in the nature of its fibres. The main stem and the right branch show conspicuous pathological lesions. The lower portions of the right branch show that this structure is partially atrophic. The left branch is small above and larger below; it presents no serious lesion. A complete transverse lesion is therefore absent. The area of the muscle in the bundle, seen in the cross section, is seriously reduced by connective tissue formation and by the presence of the sinuses described. There is no evidence of an acute or subacute process. The lesions are manifestly chronic.

SUMMARY.

A case of Adams-Stokes' syndrome is described in which bradycardia occurred from time to time for nearly six years. An electrocardiographic examination thirteen days before death revealed complete heart-block. The patient died comatose; old inflammatory lesions were found which seriously compromised but did not completely divide the main stem and right branch of the auriculo-ventricular bundle.

AURICULAR FIBRILLATION AND COMPLETE HEART-BLOCK. A DESCRIPTION OF A CASE OF ADAMS-STOKES' SYNDROME, INCLUDING THE POST-MORTEM EXAMINATION.

By ALFRED E. COHN,

*(From the Hospital of The Rockefeller Institute for Medical Research,
New York)*

AND

THOMAS LEWIS.*

*(From the Cardiographic Department, University College Hospital Medical
School, London.)*

CLINICAL OBSERVATIONS.

The history and the condition of the patient who forms the subject of this communication have been described in three previous articles. The original account is to be found in Mackenzie's paper (8). More detailed accounts have been published by Lewis and Mack (7), and by Lewis (6).

It may be well briefly to recapitulate these reports.

M. M. was born in 1865; he contracted syphilis in 1887. In 1894, he had his first attack of syncope and had suffered from more or less prolonged attacks of loss of consciousness, sometimes accompanied by convulsions, up to the time when he was first examined. The first record of slow pulse rate dates from 1906, but his detailed history leaves little doubt that it had been present for a longer period.

He was seen by Dr. Mackenzie in November, 1908, when the first tracings were taken, and his pulse rate was then about 30 to the minute. He was under continuous observation from this time until the date of his death and was an inmate of a very large number of hospitals and infirmaries in London. The condition of the

* Working under the tenure of a Beit Memorial Research Fellowship.

heart during the whole of the three years was almost constant. The ventricle generally beat quite regularly, at a rate of about 32 beats per minute, though from time to time the rhythm was disturbed by premature ventricular contractions. A total absence of any sign of coördinate auricular contraction throughout, and the replacement of these signs by those which, as we now know, characterise fibrillation of the auricle was a remarkable feature of the case.

The diagnostic features as they were summed up in the last report are as follows:—

1. *The evidence of complete heart-block.*
 - A. A history of syphilis.
 - B. The occurrence of fits accompanied by cessation of the ventricular action.
 - C. The persistence of a slow ventricular action in the intervals, at rates known to be characteristic of an independent ventricular rhythm.
 - D. The absence of compensatory pause after the premature ventricular beats.
2. *The evidence of auricular fibrillation.*
 - A. The complete absence of *a* waves in the jugular curves in scores of observations.
 - B. The complete absence of *P* variations in the electrocardiograms (Fig. 4).
 - C. The presence of the characteristic oscillations of auricular fibrillation in the electrocardiograms (Fig. 4);* and, as may be now added, the occasional presence of rapid undulations in the venous curves during the diastoles.
 - D. The determination that the electric oscillations were maximal when leads were taken directly from the chest wall, the electrodes being fixed in the vicinity of the right auricle.

It was upon this evidence that the conclusion was based that the two conditions, heart-block and auricular fibrillation, were present in one and the same patient.

In regard to the general course of the patient's illness between 1908 and 1911, it is only necessary to state that apart from slow

* For the figures, reference should be made to the original descriptions.

and progressive weakening, there was no change, and that abundant records of the pulse and venous curve were taken in Dr. Mackenzie's clinic up till the day preceding death.

The Nature of the Terminal Fits.

The fits observed during the last days of the patient's illness were not dissimilar to those previously recorded, with the exception that no movements of the veins of the neck were seen during the long periods of asystole which accompanied them. They have been described for us by Dr. Silberberg to whom we are indebted for the following observations.

"The attacks of unconsciousness and the mild convulsions commenced at 5 o'clock on the morning of July the 6th, 1911. They ceased at 9 a. m., but returned at 11 a. m., and continued at short intervals till 1 p. m. Between 1 p. m. and 2.30 p. m. he was free from them. From this hour until death occurred (the morning of the 8th) there was a similar repetition of relapse and recovery."

"The attacks were of varying duration; lasting for a few seconds to twenty or thirty seconds. They were accompanied, one and all, by a lapse of the ventricular beats, readily observed at the prominent apex beat. The onset of unconsciousness was gradual, and the patient was aware of the impending attack, being conscious that his heart has temporarily ceased to beat. He became restless; he groaned and uttered words of complaint. In a few seconds he could not be roused, the eyes rolled upwards and deviated to the right; the pupils dilated fully, and the corneal reflex was lost. Cyanosis of the face, already present, deepened to lividity; the breathing became stertorous and air was sucked in vigorously, his cheeks sinking deeply between his edentulous gums. Much flatus was passed. After twenty seconds of unconsciousness, epileptiform manifestations appeared; the convulsions started in the face, the arms became rigid and spasmodic flexor movements appeared. The lower limbs showed no convulsive movements. A single beat of the heart, during the fit, lightened the degree of unconsciousness. Two or three beats brought him to a dazed condition and, after a few more beats, he rapidly recovered, conversing rationally, though necessarily showing exhaustion. The first beat of the recovery was

usually a weak one, the ensuing beats were more forcible. During the periods of comparative lucidity he complained of aching and sore feelings all over, more especially in the limbs. He was too feeble and exhausted to move. Pain, relieved by eructation, was present over the upper abdomen. On several occasions he vomited a pint or more of greenish fluid, an incident which seemed to afford relief."

The patient died in a fit on the morning of July the 8th. He became suddenly and deeply cyanosed, the face and arms showed the usual convulsive movements, but he failed to recover from the attack.

During the period of "status epilepticus," continuous tracings were taken by Dr. Silberberg from the apex beat. The rigidity of the arms and neck rendered further graphic record impossible; the ventricular pulsations were the only prominent movements. The whole length of a long curve of 21 minutes' duration is included in the accompanying diagram (Fig. 1), in which each beat has been accurately placed; (the error is nowhere greater than one-twentieth of a second). The rate at any point may be calculated from the vertical lines which are separated by two-second intervals. Two strips of the actual curve are published as examples of the observations (Fig. 2 and 3).

The events which are portrayed in this diagram are of very considerable interest. The usual rate of the rhythm of the ventricle for this patient was 32 per minute. This rhythm appears from time to time in the chart, and the rate is either 30 per minute or somewhat less, namely 27; thus it is seen over the whole of the lines, *h*, *j*, *k*, *l*, and *m*, and over the greater part of line *e*. The rhythm is not quite regular for premature beats appear from time to time. These beats are separated from those which precede them by a second or a little more or less; occasionally two or three premature beats succeed each other (line *l* and line *d* directly after the pause), each following at about the same interval. From time to time they recur so frequently as to form a new rhythm, varying in rate from 60-80 per minute. It is the relation of these relative tachycardias to the periods of asystole which is so important. *The tachycardia, except where it is of brief duration, as in line "e," is invariably followed by a prolonged pause; and none of the prolonged pauses*

of the curve occur except immediately at the termination of a period of relative tachycardia.

The meaning of this phenomenon is quite clear. We have a clinical repetition of a phenomenon which has been studied experimentally and in detail by Erlanger and Hirschfelder (4). When a slow ventricular rhythm is developed as a result of bundle section and a new and interrupting rhythm of faster rate is established, the

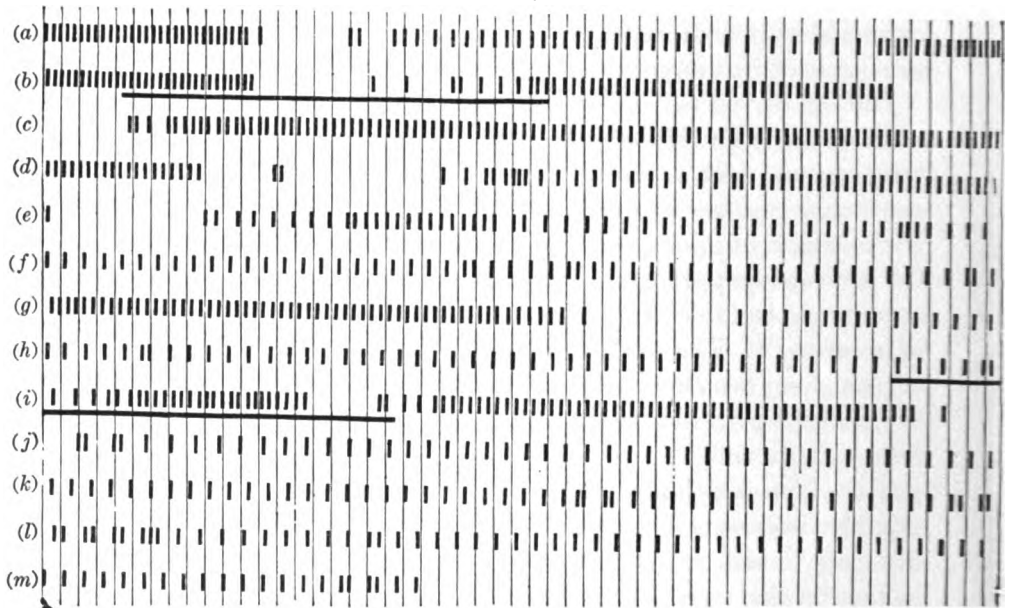


Fig. 1. A diagram compiled from a continuous curve of the apex beat, the duration of which was 21 minutes. The diagram reads from left to right, following consecutive lines. Each beat of the original curve has been charted on a large scale and the chart has been reduced subsequently photographically. The vertical lines are placed at two-second intervals. Two pieces of the original curve are reproduced in Fig. 2 and 3; the corresponding portions of the diagram are marked by means of heavy horizontal lines drawn beneath them. The relation of the (relative) tachycardial periods to the long asystolic intervals is very clearly shown.

new rhythm takes precedence to the old, and the latter passes into a condition of temporary abeyance. The cessation of the new rhythm is marked by a period during which physiological impulse formation in the ventricle is dormant and its awakening is gradual; hence the

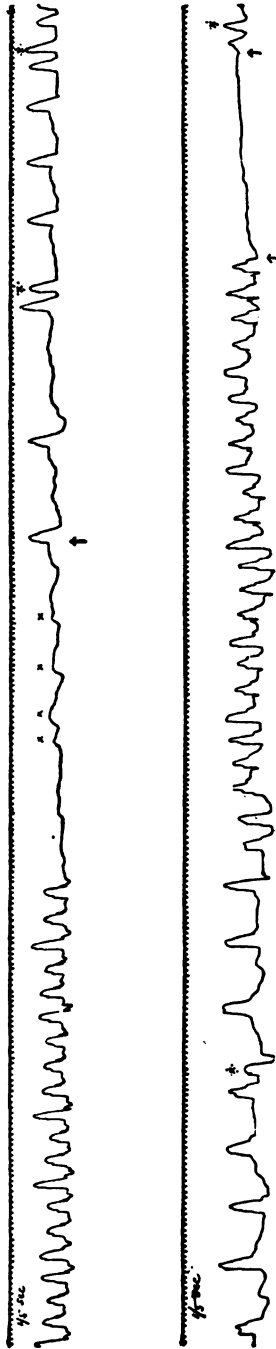


Fig. 2 and 3. Portions of the original curve from the apex beat. The time-marker is in one fifth seconds.

Fig. 2 shows the termination of a period of relative tachycardia; it is followed by a pause of 13 seconds (the pause is interrupted by movements of the lever which were due to restlessness of the patient). The pause is followed by a slow but accelerating rhythm.

Fig. 3 shows the commencement and termination of a short period of relative tachycardia; it commences in a premature beat and ends in a long pause of 8 seconds' duration. Premature beats are marked by asterisks in the figures.

long pause which follows the ending of the new rhythm. The nature of the fits, and the underlying cause in this patient, is consequently apparent. The long asystole, or "stoppage" as Erlanger has termed it, is brought about by an interference with the natural production of the idio-ventricular rhythm, as a result of the successive appearance of premature beats. In experiment the longest pause is generally followed by other pauses, which, though considerably longer than those which separate the beats of an established rhythm, are shorter than those which precede them. The same events are seen in this clinical case, though they are more irregular than are the experimental instances.

POST-MORTEM EXAMINATION.

The post-mortem was carried out by one of us at Mount Vernon Hospital on the 8th of July, 1911, a few hours after death. The body was that of a well developed and well nourished individual. Rigor mortis was present. There was slight œdema of the ankles. The conjunctivæ and skin had a yellowish tinge. An old and dense scar, 4 cm. in diameter, was noted above the right knee. Four other scars, each about 2 cm. in diameter and having a somewhat serrated margin, lay together over the left iliac crest. The external and internal jugular veins were a little dilated. The costal cartilages were not calcified.

Macroscopic Examination.

The *tonsils* were fibrosed. The *tongue* and *palate* were normal. The mucous membranes of the *larynx* were yellow and thickened and showed some superficial petechiæ. The *trachea* contained a little frothy and bloodstained mucus. There were a number of dilated veins at the cardiac end of the *œsophagus*. The *stomach* was more dilated than normal and its mucous membrane was deeply congested. The *intestines* and colon were not distended. The mucous membrane was injected.

The *pleuræ*. On the right side, a transverse shelf of very strong adhesions, between diaphragm and parietal pleura, isolated the lower 12 cm. of the cavity. The lung lay entirely above it, but the space communicated with the main cavity by a small aperture in

front. Isolated, thin but strong adhesions united the lung and parietal pleura; they were thicker and more numerous at the apex and along the posterior margin. No fluid was found in the sac. On the left side and on a level with the heart, the lung was not visible from the front. The whole pleural space was obliterated by soft, spongy adhesions resembling connective tissue.

The *pericardium*. The pericardium was bound to the two pleural membranes, and to the chest wall in front, by soft adhesions of a character similar to those found in the left pleura. When fully exposed and viewed from the front, the pericardial sac and its contents filled the whole of the lower portion of the left pleural cavity. At the level of the fourth interspace, the right margin lay 7.5 cm. from the middle line. At the level of the sixth cartilage, the margin lay 11.5 cm. from the middle line. The apex lay in the anterior axillary line in the sixth interspace. The pericardial cavity was completely obliterated by abundant adhesions; the two layers were fairly readily separable. The adhesions were firmer over the right auricle, and especially along the line of the sulcus terminalis, but the entire surface of the pericardium was roughened by the adhesions, which were old and dense. From the *A-V* groove to the apex along the anterior interventricular groove, the ventricle measured 16 cm. A similar measurement on the posterior surface was 12.5 cm. The width of the heart along the posterior *A-V* groove was 13 cm. The right ventricle occupied the greater part of the anterior surface.

The *heart*. The heart weighed 899 grammes. The surface of the heart presented a considerable increase of fat deposit. The fat around the auriculo-ventricular groove was oedematous. There was little or no blood in the ventricles, but there was an excess of it in the great veins and auricles. The right auricle showed extreme dilatation, more especially the right auricular appendix. The cavity was about the size of a closed man's fist. The *tænia terminalis* in its upper extremity was hypertrophied, as were also the muscoli pectinati, but the posterior wall of the right auricle was very thin. The endocardium was whiter than normal and seemed to be especially thickened below the orifice of the coronary sinus.

The tricuspid ring measured 13 cm. in circumference and readily admitted four fingers in line. The edges of the valve were slightly

thickened but not hardened, and apart from slight matting together, showed no abnormality.

The right ventricle was slightly dilated. Its walls and trabeculæ were hypertrophied. The muscle was brown, soft, coarse and friable. The wall measured 9 mm. at the base at the right border, 4 mm. at the apex, and 6 mm. at the conus arteriosus. On the trabeculæ and more especially at their origins and points of division there were irregularly shaped oval and linear thickenings of the endocardium. They were cream coloured, dense in consistency and circumscribed. These were elevated as much as 2 mm. above the surrounding surface and were from 1 to 2.5 cm. in length. On the endocardium of the conus arteriosus, 15 mm. from the pulmonary valve there were areas of a similar nature but more extensive, one measuring 20 and another 25 mm. in length. Their outline was very irregular. The cusps of the pulmonary valve were fenestrated and the *corpora Arantii* were slightly thickened.

The left auricular appendix was bound down by adhesions and was noticeably smaller than the right. It measured 3.8 cm. as opposed to 8 cm. for the right, the measurement being made from the middle of the *tænia terminalis* to its apex. The wall of the auricle was dilated and moderately hypertrophied; it measured 6 mm. The endocardium was white and thick, but smooth. The mitral ring admitted three fingers in line. The edges of the mitral valve were thickened and smooth and not hardened. In the attached margins there were no calcareous deposits.

The wall of the left ventricle was dilated and hypertrophied and measured 17 mm. at the base, 22 mm. at the level of the papillary muscles and 9 mm. at the apex. The outflow tract below the root of the aorta was dilated. The wall of the left ventricle was thin at the left border, measuring only 5 mm., 4 cm. above the apex. On this portion of the surface of the heart, the adhesions were very dense. The main branch of the coronary artery to this portion of the heart was not occluded. In places there were yellowish streaks in the muscle. Elsewhere there were numerous scars. The enlarged papillary muscles showed white subendothelial nebulæ. The endocardium was smooth on the whole, but moulded on the trabeculæ and also at their junctions, cream-coloured plaques were found

like those in the right ventricle. They were more numerous on the left side. The posterior aspect of the posterior papillary muscle was entirely encased by such material. In the outflow tract, directly under the anterior portion of the membranous septum, a similar thickening was found, elevated 2 mm. above the level of the endocardium, and measuring 1×2 cm. The other plaques of similar nature varied in length from a few millimetres to several centimetres. No calcareous deposits could be felt in or about the septum membranaceum. At the upper portion of the left ventricle, at the level of the *A-V* ring and at the right half of the cavity was an *intracardial aneurysm*. Its exact position (Fig. 5) was to the right of and behind the junction of the two flaps of the mitral valve and consequently to the left of and below the origin of the aortic valve, more especially its right posterior cusp. The aneurysm was shaped like a portion of a sphere. Its diameter was 33 mm. The posterior wall of the aneurysm was formed by the posterior wall of the ventricle, and its level was that of the coronary sinus. The coronary sinus was compressed from before backwards but its lumen was not compromised, while the branches of the coronary artery, though small, were not occluded. The right wall of the aneurysm was formed by the septum between the left ventricle and right auricle and corresponded in part to the insertion of the mesial flap of the tricuspid valve. The wall of the aneurysm was calcareous but smooth and showed no thrombi. Pressure in the aneurysm was directed backwards, upwards and to the right and was exerted on the end and the opening of the coronary sinus, and also on the septum between the two sides of the heart so that this was protruded into the cavity of the right auricle (see Fig. 5). The involved area corresponded, when seen from the right auricle, to the wall lying between the opening of the coronary sinus and the septum membranaceum. It is just in this situation that the auriculo-ventricular node and the beginning of conduction system is usually found. It was presumed that the aneurysm might have damaged these structures. Examination of Fig. 8 will show that this was not the case.

The lunulæ of the aortic valve were thickened and so were the *corpora Arantii*, but the valve presented no other abnormality.

There was some atheroma in the sinuses of the *Valsalva*. Atheroma and athero-sclerotic patches were found along the course of the coronary arteries. These did not seriously narrow the lumina of the vessels anywhere. The foramen ovale was closed.

The *arteries*. The pulmonary artery showed occasional atheromatous patches and was pinkish-yellow in colour. The thoracic aorta was bile stained, the endothelium seemed to be a little œdematous and there were scattered patches of atheroma. The abdominal aorta was extensively degenerated. Atheroma was advanced; œdema and ulceration were present. The coronary arteries presented little thickening; the coronary sinus and the cavities of the heart were free of ante-mortem clot.

The *lungs*. The right lung weighed 560 grammes, the left 525. The tissue was deeply pigmented, emphysematous and a little œdematous. Otherwise these organs were normal. The bronchial lymph nodes were black. Neither these nor the mediastinal nodes were enlarged or fibrosed.

The *liver* weighed 1,236 grammes. It was small and very hard. A number of firm, isolated adhesions united it to the diaphragm. The surface was irregular and showed a mild degree of hobnailing. One or two large superficial scars were present. The section was mottled red and greenish-yellow. The red areas were depressed. Fine, pink trabeculæ of fibrous tissue were clearly visible throughout large areas of the organ. The tissue was very tough. Glisson's capsules seemed more fibrous than usual. The liver appeared to be fatty, fibrous, congested and somewhat bile stained. The gall bladder was small and the wall was thick.

The *spleen* weighed 640 grammes. The surface was adherent to the diaphragm in several places. A number of small, irregularly shaped, cream-white masses were present on the surface and projected from it. The organ retained its shape. The section was dark, the tissue tough; the Malpighian capsules were not prominent. There seemed to be an increase of fibrous tissue.

The *pancreas* was larger than normal and consisted chiefly of mottled pink and yellow areas, in which the gland substance could be traced only from place to place.

The *kidneys* weighed 176 and 192 grammes respectively. The

capsules were non-adherent. Apart from the conspicuousness of the glomeruli and slight general congestion and increased firmness, they seemed to be normal.

The *suprarenals* appeared to be normal. The cortex was dark brown, and the medullary substance light grey.

The *testicles*. Both tunicae vaginales contained about a drachm of yellow fluid. On the right side the testicle was normal, and there was a varicocoele. The left testicle was small and fibrosed.

The *peritoneum* was smooth. There was about a half-pint of yellow fluid in the cavity.

The *brain*. The calvarium was normal. The pia-arachnoid membrane was oedematous; the superficial veins were congested. The basal and cortical arteries were normal. The brain showed no abnormality on section. The venous sinuses were empty.

The *vagus and sympathetic nerves* presented no abnormality.

Microscopic Examination.

All the pieces excised for microscopic examination were fixed in Müller-formol (9:1). The heart, medulla oblongata, pons and vagus nerves were fixed in the same fluid. The technique of examination of the heart was the same as that used and already described in this *Journal* (1). On account of the difficulty which would have been experienced in cutting sections of the septum of the heart with the aneurysm in place, it was decided to shell out this structure. This proceeding was accomplished successfully. No incisions were necessary; blunt dissection sufficed to enucleate it in one piece. The description given below (see Fig. 5) will show that no injury whatever was sustained by the structures of interest in this study. The following tissues were excised for examination:—

- (1) The cavo-auricular junction, bearing the sino-auricular node.
- (2) A portion of the septum between the two halves of the heart, bearing the auriculo-ventricular node, main stem and branches.
- (3) A piece of the left ventricle.
- (4) Four pieces of the aorta, two thoracic and two abdominal.
- (5) Two pieces of the liver.

- (6) A piece of the pancreas.
- (7) A piece of the spleen.
- (8) Two pieces of the kidney.
- (9) The medulla oblongata and pons Varolii.
- (10) The two vagus nerves.

The *cavo-auricular junction, bearing the sino-auricular node*. A piece of the superior vena cava was left attached so that Wenckebach's bundle could be examined. Apart from the changes which occurred in the cardiac muscle fibres in this case, no pathological lesions were found. The amount of connective tissue was not greater than elsewhere and there was no evidence of acute inflammation. The muscle fibres throughout those portions of the heart examined were much larger than normal. There was no fragmentation of the muscle and no granular degeneration. The transverse striation was clear and the nuclei well stained. In many of the muscle fibres there was an unusually large space about the nucleus which was clear of muscle fibrils, so that they resembled Purkinje cells. They had no definite arrangement, and were so scattered as to make it impossible to regard them as forming a system. The spaces about the nuclei contained no pigment. The nuclei themselves varied very much in size and shape, but for the most part they were much larger than normal. Their margins were usually irregular and the distribution of chromatin was uneven. Here, as elsewhere throughout the heart, connective tissue of a loose areolar nature was increased between the fibres and numerous scars of connective tissue were found. The smaller blood vessels were abnormal. The adventitia was but little thickened but there was thickening of the muscle of the media. The position of the internal elastic membrane showed an increased amount of connective tissue which sometimes extended into the intima. In a few places, loose connective tissue was seen invading the media from the adventitia. Vacuoles appeared frequently in the muscle fibres of the vessels. The intima was hyperplastic in many places, and the lumen often seriously reduced in diameter. A few small vessels were found to be occluded and others presented but a slit-like channel. The elastic tissue throughout the heart stained poorly or

not at all, and was in marked contrast to that found in the kidney of the same case, which was used as a control.

The sino-auricular node was seen at a level below that at which the cavo-auricular junction was formed. The angle of junction lay, in fact, 3 mm. above the upper extremity of the node.* The node could not be said to lie in the wall of the superior vena cava. At the anatomical junction between the superior vena cava and the auricle, there was complete separation of the muscular systems of both by fat and by some connective tissue. It was in this fatty tissue that the node was first seen, lying much nearer the vena cava than the auricle but quite separate from it. From its upper extremity to its end, it measured 21.55 mm., the measurement being computed from the thickness of the sections multiplied by the number in which it was found. The upper extremity of the node lay, as has been said, nearer the superior vena cava, while a little lower down it was situated nearer the auricular muscle. It was found close to the pericardium throughout, except at its upper extremity. Here it was 3.5 mm. from the surface, but a little lower (1.5 mm.), it was 1.5 mm. deep and maintained this depth (0.75 to 1.5 mm.) throughout its extent. The shape of the node varied constantly in its course. In its highest level it was roughly triangular, the apex of the triangle pointing toward the pericardium. A little further down, the node was circular (Fig. 6). But the greater portion of it (66 %) had an elongated shape, the long axis in cross section being parallel to the pericardium (Fig. 7). The greatest length of this long axis was 5.5 mm. and its width at the corresponding level varied from 1.0 to 0.25 (See Table I).

The upper third of the node was entirely destroyed by connective tissue. This was dense and probably old (Fig. 6). Fat tissue occupied the area at the sides of the node, between it and the pericardium, and was also found within its structure. Except in one or two places, where there were round cells, there was no evidence of a recent or acute inflammation. Below this level, that is, in the lower two thirds, muscle tissue appeared in the node and rapidly in-

* Koch (5) has shown that in the hearts of dogs, an extension of the sino-auricular node reaches 2 mm. upward on the wall of the superior vena cava, but that the node can be recognized 0.75 to 1 mm. above the cavo-auricular angle.

TABLE I.

Slide		Depth.	Length.	Width.
122	3.5 mm.....	0.75 × 1.5	mm.
"	134 2.0 mm.....	1.00 × 3.0	mm.
"	141 mm.....	1.00 × 2.0	mm.
"	155 1.5 mm.....	2.00 × 2.0	mm.
"	181 1.0 mm.....	2.00 × 1.0	mm.
"	199 1.0 mm.....	2.00 × 1.0	mm.
"	226 1.0 mm.....	1.50 × 1.5	mm.
"	257 1.0 mm.....	1.50 × 1.5	mm.
"	284 1.0 mm.....	2.50 × 1.0	mm.
"	292 0.75 mm.....	3.00 × 1.0	mm.
"	310 0.75 mm.....	4.50 × 0.5	mm.
"	324 0.75 mm.....	5.00 × 0.5	mm.
"	344 0.75 mm.....	5.50 × 0.5	mm.
"	351 1.00 mm.....	5.50 mm.	
"	358 1.00 mm.....	3.0 × 0.25	mm.
"	364 1.00 mm.....	3.5 × 0.5	mm.
"	384 1.25 mm.....	3.0 × 0.25	mm.
"	393 1.50 mm.....	2.25 × 0.25	mm.
"	402	1.50 mm.	
"	416 1.50 mm.....	0.50 mm.	

creased in amount, but the amount of connective tissue was probably always more than normal (Fig. 7). The increasing amount of muscle tissue occurred at the anterior portion of the node, while the posterior portion remained quite sclerosed. The node was at almost every level in direct relation with nerve trunks. Ganglia were found, as were also nerves which contained ganglion cells. About some of these there was dense connective tissue. The node contained an artery and in some sections two. In some parts of its course, the artery showed a distinct endarteritis. A small artery at one level was entirely occluded and canalized.

The *auriculo-ventricular system*. A block of tissue, including the interauricular and interventricular septum, was excised. The upper margin ran parallel with the upper edges of the aortic cusps, the lower margin was parallel with this, two to three centimetres below the membranous septum; the anterior margin was at the anterior extremity of the septum, and the posterior margin was the posterior wall of the heart itself. The sections were cut parallel with the upper margin. The entire site of the aneurysm already described was included in the block. The bed of the aneurysm was

found to be a connective tissue structure, of a dense fibrous nature, which looked old. In a few places near the wall there were collections of lymphocytes. This was the only evidence of recent or acute inflammation. The bed of the aneurysm, which was also the interauricular septum (see Fig. 5 and 8), was very much thinned, convex towards the right auricle and contained no muscular tissue. The entire septum was consequently converted into a wall composed of fibrous tissue. Examination showed that the removal of the calcareous portion of the aneurysmal sac had involved the loss of no structure necessary for this study. The septum contained no muscular structures between the coronary sinus behind and the central fibrous body in front. Muscle fibers survived above the level of the aneurysm rather high above the auriculo-ventricular groove, but they did not descend to the auriculo-ventricular node. There was consequently no connection between the auricular muscle and the node; the auriculo-nodal junction did not exist. Except for the muscle tissue of the auriculo-ventricular system, to be described presently, the entire interauricular and interventricular septum in the neighbourhood of the aneurysm, the central fibrous body and the septum membranaceum were replaced by dense old connective tissue, poor in nuclei; and in some places, notably in the posterior wall of the heart and at the root of the aorta, by fatty tissue. That portion of the interventricular septum, in which the upper portions of the right and left branches of the conducting system are usually found, was also replaced, for 2 cm. anterior to the membranous septum, by connective tissue of the kind described. Muscle tissue was seen only in the lower parts of the block. In the sections the coronary sinus was seen to be compressed from before backwards. That wall of the sinus which was directed toward the cavity of the auricle contained a thick layer of loose connective tissue. The cardiac muscle tissue in this situation was thinned and was infiltrated with large amounts of dense connective tissue. A number of vessels were compressed and showed advanced endarteritis to the point of occlusion.

The auriculo-nodal junction, as has been said, did not exist. The node was, however, a well developed structure and showed the familiar interlacing arrangement. It followed the usual appearance

of this structure also in the number, shape and size of the nuclei, the fineness of the fibres and the arrangement of the fibrous tissue. It was a normal node. At one portion a few small, dense masses of connective tissue were present but they did not appear sufficient in size or number to be considered pathological. The artery to the node appeared in the lower regions only. Its adventitia was thick and there was much connective tissue in the intima. At still lower levels the lumen was contracted on account of endarteritis. Passing forward from the node (Fig. 8), the main stem of the *A-V* bundle progressed normally through the septum membranaceum for about 2 mm. when it ceased suddenly in the dense connective tissue of the septum already described. About 3 mm. farther along, the main stem was again identified. A hiatus of about 3 mm. was thus caused between the *A-V* node and the main stem, in the upper portion; of about 7 mm. at the lower; and of 12 mm. at the lowest level. The main stem was then followed through the septum membranaceum about 3 mm. Its anterior end was terminated by the dense connective tissue of which the septum ventriculorum was composed. The division of the main stem into the right and left branches was nowhere seen. This portion of the system, as well as the origin of the branches, was completely destroyed by the sclerotic process. The right branch was not identified at lower levels, but the left branch was clearly seen 6 mm. below the point at which the main stem disappeared. The failure to identify the right branch was due, no doubt, to the fact that the right half of the interventricular septum was sclerotic at levels lower than the left half. The left branch of the bundle did not partake of the hypertrophy of the rest of the heart. The nuclei of the conduction fibres were slightly irregular but the change from the normal shape and structures was much less than that seen in the intrinsic cardiac muscle. The distinctiveness of the nuclei of the conducting muscle was recognised here as well as in other hearts. The endocardium which lay over the left branch was especially thick, the thickening being due to fibrous changes in the lining membrane of the heart. The smooth muscle of the endocardium showed a normal development.

The *aorta*. The cusps of the sinuses of *Valsalva* were sclerotic, and this sclerosis involved the junction of the cusps especially. The

aortic wall contained an increased amount of connective tissue. The thoracic aorta showed moderate thickening of the intima, the elastic tissue of which was hyperplastic. The medial layer was practically normal. Occasionally large plaques of the intima occurred, which showed degenerated and oedematous loose fibrillar connective tissue. They contained no calcium, cholesterin, or debris. The abdominal aorta showed areas where there were lymphocytic and rarely leucocytic collections in the intima. In a few places the media where it adjoined the intima was degenerated. Here the nuclei were not stained and the tissue looked hyaline. Elsewhere the media showed an increased amount of connective tissue. The intima was unequally thickened and showed distinct plaques. The plaques were hyaline, non-cellular, and in a few, large collections of needle-like vacuoles were seen, which in the recent state had been occupied by cholesterin crystals. In other portions of the intima there were cellular elements, lymphocytes, leucocytes and plasma cells. There was no deposit of calcium. Another section showed necrotic plaques which were in a pre-ulcerative stage. Here the degenerative changes in the media were more advanced, in that the nuclei did not stain and the tissue had assumed a hyaline appearance.

The *liver*. The capsule was very thick and in places shaggy. The thickening was for the most part due to an increased amount of fibrillar connective tissue, of normal appearance. In other places the thickening was due either to destruction or to partial atrophy of the underlying liver cells. The central veins were dilated, the surrounding liver cells were atrophic, and in many there was advanced fatty degeneration. A number of these cells showed brown pigment. In these areas a number of acini were compressed. The capsules of Glisson were normal.

The *pancreas*. There was increased interstitial fatty tissue and also some increase in the amount of connective tissue.

The *spleen*. The capsule was much thickened by dense masses of connective tissue. The substance of the gland was congested; and there appeared to be more than the normal amount of lymphatic tissue in it.

The *kidneys*. The glomeruli were distinctly more congested than

were other parts of the organ. Adjoining the capsule, there were a number of small areas containing lymphocytes and others where there was an increase of connective tissue. The vessels were normal. There was a hyperplasia of the internal elastic membrane, probably normal at this period of life.

The *medulla oblongata* showed no abnormality. It was examined in serial sections.

The *right vagus nerve* showed no abnormality. Many ganglia were seen incorporated in its course. A small nerve (presumably the sympathetic) was seen running parallel with the vagus, into the interstitial tissue of which a hæmorrhage had taken place. The *left vagus* was like the right and in the section a small nerve containing a hæmorrhage was also seen.

Summary.—(1) The medulla oblongata showed no gross lesion. The vagus nerves were normal, but parallel with each nerve there was a small one containing a hæmorrhage. (2) The heart was hypertrophied in all its cavities. There was an aneurysm in the right upper portion of the left ventricle. There was partial sclerosis of the septum ventriculorum and complete sclerosis of the septum membranaceum. The myocardium contained numerous scars. There was practically no acute inflammation. The sino-auricular node was in part destroyed, being replaced by connective tissue. The main stem of the auriculo-ventricular bundle was divided from the auriculo-ventricular node by sclerotic tissue; and the distal end of the main stem, its point of division and the upper parts of both branches were destroyed by the same process. The arteries of the heart showed hypertrophy of the media, degeneration of their muscle fibres, and hyperplasia of the intima causing either partial (the more common lesion) or complete obliteration of the lumina. The aorta showed athero-sclerosis. (3) The liver showed chronic congestion, as did also the spleen, pancreas and kidneys.

RELATION OF LESIONS AND HEART MECHANISM.

We do not propose to discuss the question of auricular fibrillation and its morbid anatomy at any length. The findings in this case, the partial destruction of the sino-auricular node and the scattered

fibrosis in the auricle, conform with those which have been found by a number of other writers.

The case was remarkable clinically for the presence of definite signs of auricular fibrillation, while *the ventricular action instead of being rapid and irregular*, as is usual in such cases, was *slow and regular*. The opinion was held, and is still held, that a regular action of the ventricle is never associated with auricular fibrillation, except when complete functional dissociation of auricle and ventricle, so far as conduction is concerned, is present; and it was felt that as the ventricular action was persistently slow and regular, a lesion accounting for this action would be found, which was comparable to the lesions discovered at autopsy when ordinary dissociation of auricular and ventricular rhythms occurs. This expectation has been fully realised by the examination of the heart in this unique case. Complete division of the junctional system occurred at two levels at least. The post-mortem findings confirm the conclusion that if, in a case of auricular fibrillation, the action of the ventricle is regular, there is complete functional separation of the two chambers, so far as the conduction of impulses is concerned. The case is, so far as we know, the only one of its kind which has been recorded.

But there is another matter in connection with it of considerable interest. The post-mortem examination shows a lesion at the division of the bundle, and *destruction of the upper ends of both the branches*. Now when there is a single lesion of the main stem, the electrocardiogram of the ventricle retains its original form, consisting of the normal *Q*, *R*, *S* and *T*, or *R*, *S* and *T* variations. When one or other branch is divided, anomalous electrocardiograms are produced, which as Eppinger, Rothberger and Stoerk (2 and 3) have shown, are distinctive of the lesions in question. In this clinical instance both branches were destroyed and it might be anticipated that, as a result, the ventricular electrocardiogram would be considerably modified. One of the original curves is shown in Fig. 4; it was taken from lead *II*. Two ventricular beats are seen; *R* is small, *S* is deep and *T* is tall, but the chief feature of the curve is *the breadth of "S."* The total duration, from the com-

mencement of *R* to the end of *S*, is approximately one-fifth of a second.

There is but one published electrocardiogram from an experiment in which both bundle branches had been cut. It is given by Eppinger and Rothberger (2) (Fig. 8 of their paper). The curve is almost identical with that now published; *R* is short, *S* is deep and *T* is full; but again the chief feature is the duration of *S*. The



Fig. 4. (Reproduced from *Heart*, Vol. 1, p. 306, Fig. 18). An electrocardiogram, showing auricular fibrillation and the slow and peculiar ventricular beats which are considered to be associated with the action of a heart, in which both bundle branches have been destroyed. Note the duration of *S*. The time marker is in one-fifth seconds.

clinical and pathological observations are thus in the most complete accord. It seems from the comparison that division of both branches of the bundle may be diagnosed clinically.

SUMMARY.

1. The pathological report of a patient previously described as exhibiting auricular fibrillation and complete heart-block is now given. Complete division of the bundle was found, and lesions compatible with auricular fibrillation were seen.

2. The observations support a former conclusion that, when auricular fibrillation is associated clinically with a regular action of the ventricle, impulse conduction from auricle to ventricle is in abeyance.

3. A lesion is also described which divided both branches from the main stem of the bundle and from each other; the electrocardiograms were of the form seen by Eppinger and Rothberger to follow a similar experimental lesion.

4. The lesions in the heart were of syphilitic origin and included

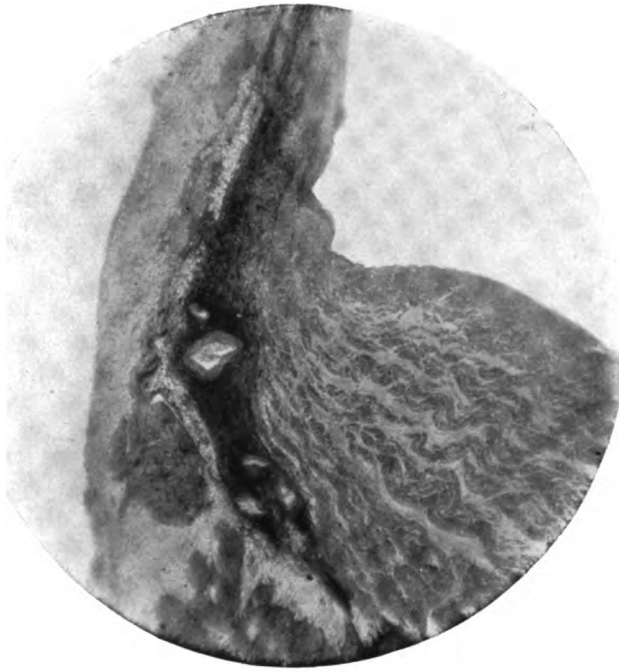


Fig. 7.

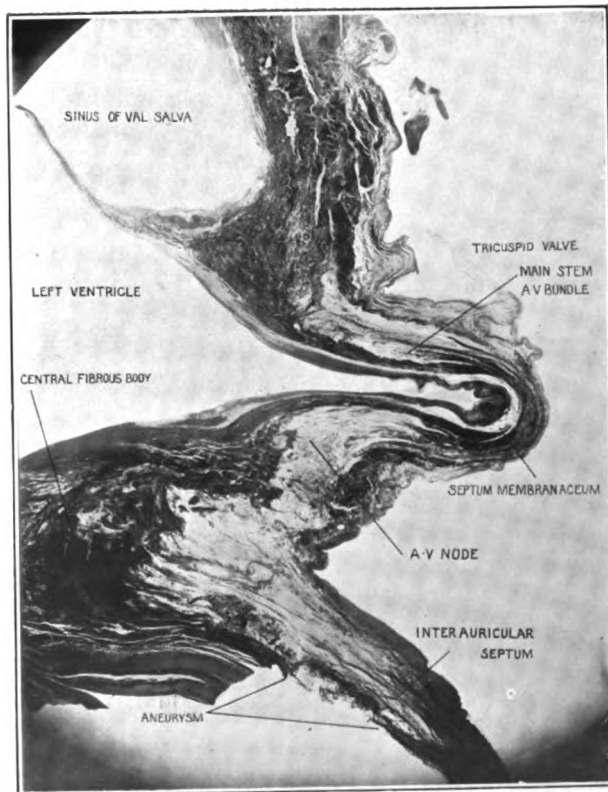


Fig. 8.

a septal aneurysm pointing from the left towards the right side, *i. e.*, from the direction of greater to that of lesser pressure.

5. The patient was the subject of syncopal attacks. The nature of the heart pauses, responsible for the attacks, has been shown. They followed periods of relative tachycardia, resulting from new impulse formation in the ventricle. This observation is exactly parallel to the experimental findings of Erlanger and Hirschfelder.

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RHYTHMIC CHANGES IN THE HUMAN HEART-BEAT.

BY G. CANBY ROBINSON AND GEORGE DRAPER.

(*From the Hospital of The Rockefeller Institute for Medical Research,
New York.*)

INTRODUCTION.

The following paper discusses three cases in which the mechanism of the heart-beat changed in a rhythmic manner. These changes have been recorded by means of electrocardiograms and venous tracings, and so analyzed. In the curves from all of the cases, groups of beats are seen which the electrocardiograms and the venous tracings indicate are abnormal. These abnormal groups alternate rhythmically with heart-beats of apparently normal origin and course. They recur at regular intervals and the groups show a striking similarity to one another in each case. As the type of abnormal beat is different in each case, a separate description and discussion of each will be given and then the points common to all will be considered.

PART I. (CASE I.)

History.—The patient is a well developed boy of seventeen years, apparently in perfect health. His childhood is said to have been free from severe illness and the history contains nothing which may be considered as an etiological factor in the production of a cardiac disorder. In 1905, at the age of eleven, it was accidentally discovered that his heart was irregular. He was given strophanthus, digitalis and other drugs for about two years without apparent effect on his arrhythmia. His exercise, which had been sometimes quite violent previous to the discovery of the arrhythmia, was somewhat restricted. Except for palpitation, which occurred occasionally when his attention was directed to his heart, he has always been free from cardiac symptoms, even during active exercise. Since the arrhythmia was first noted six years ago, the pulse has

been usually irregular or abnormally rapid. At times, however, a regular normal rate has been noted for short intervals, especially during mental excitement. The numerous forms of cardiac action that have been observed will be demonstrated by the curves.

Physical Examination.—Physical examination, except for the irregular or rapid heart action, is negative. The heart is perhaps slightly enlarged, compared to the size of the chest, the apex beat being felt in the fifth space 9.5 cm. from the mid-line. Cardiac dullness extends 3.75 cm. to the right and 10.25 cm. to the left. No cardiac murmurs are heard, but an indefinite gallop rhythm at the apex is noted at times. The systolic blood pressure is 93, the diastolic 75 mm. Hg. The heart beats regularly at 121 per minute. X-ray plates and fluoroscopic examination show evidence of enlarged mediastinal glands, but no other abnormalities.

Description of Curves.—The curves have been taken at various times between March 21, 1910, and April 23, 1911, during which time the large number of observations showed that the cardiac condition remained practically unchanged.

Fig. 1, taken with the slow speed of the Jacquet sphygmocardiograph, shows the arrhythmia which was most commonly observed. The tracing from the radial artery shows that the arrhythmia consists of short alternative periods of rapid regular beats and slower coupled beats. The most striking feature of the curve is the exact correspondence between analogous parts of the various groups. Each of the three groups begins with three moderately shortened waves, the last of the three being the longest. These waves are then followed by a group in which the rate is about 125 per minute, and each group terminates with a prolonged wave. This peculiar rhythmic change in the pulse was observed many times over a period of months. It represents the usual cardiac activity in this case and varies only slightly in the number of beats which constitute the rapid or slow groups.

Figures 2 and 3, arterial and venous tracings, taken on a more rapidly moving recording surface, demonstrate further details of this peculiar rhythm. In figure 2, obtained on the same day as figure 1, the ending of one and the beginning of the next rapid group are seen. Between these groups there occurs but one normal

cardiac cycle of 0.84 seconds in duration in the arterial tracing, accompanied by the three normal *a*, *c* and *v* waves in the venous tracing. This normal heart-beat always precedes a group of rapid beats, and for purposes of identification, it is marked (X) in all the curves in which it is seen. This beat represents the first of the three moderately shortened waves which are seen in Fig. 1 to usher in a group of rapid beats. In Fig. 2 the venous tracing shows that in the next beat a small abnormal wave follows the *c* wave, marked *a*¹, falling between it and the *v* wave. During the remainder of the tracing this abnormal wave becomes more prominent and the *c* wave is no longer preceded by a normal *a* wave.

The two following beats in all the many groups recorded, the second and third beats after the normal one, have always constant characteristic features in the venous tracings. In the first of the two beats the wave following the *c* wave, marked *a*¹, becomes prominent and is distinct from the *c* wave, but is more or less fused with the *v* wave. In the following beat, the *c*, *a*¹ and *v* waves are usually partly fused, forming a large rounded or notched wave. The radial tracing accompanying these three characteristic waves in the venous tracing has also constant features. The first pulsation is always about 0.50 seconds in duration, the second somewhat shorter, provided there is any wave in the radial tracing accompanying the partly fused rounded or notched venous wave. The arterial pulse of this beat is often too weak to produce a wave in the tracing, and at best causes only a very small wave of short duration.

Following these three distinctive cardiac cycles, the remainder of the group of rapid beats show in the venous tracing large *c* waves followed by an even larger wave, marked *a*¹, falling between the *c* and *v* waves, partly fusing with the latter.

Fig. 3, obtained more than a year later, shows two groups of rapid beats which are in all essentials like those seen in Fig. 2. The two groups in Fig. 3 show a remarkable similarity to each other, especially when the details of the venous tracings are compared, and indicate how truly rhythmic these changes in the heart-beat are. Between groups I and II of this figure there are several slow beats which have not been reproduced. Here it is again seen that each group consists of the normal beat (marked X), the three

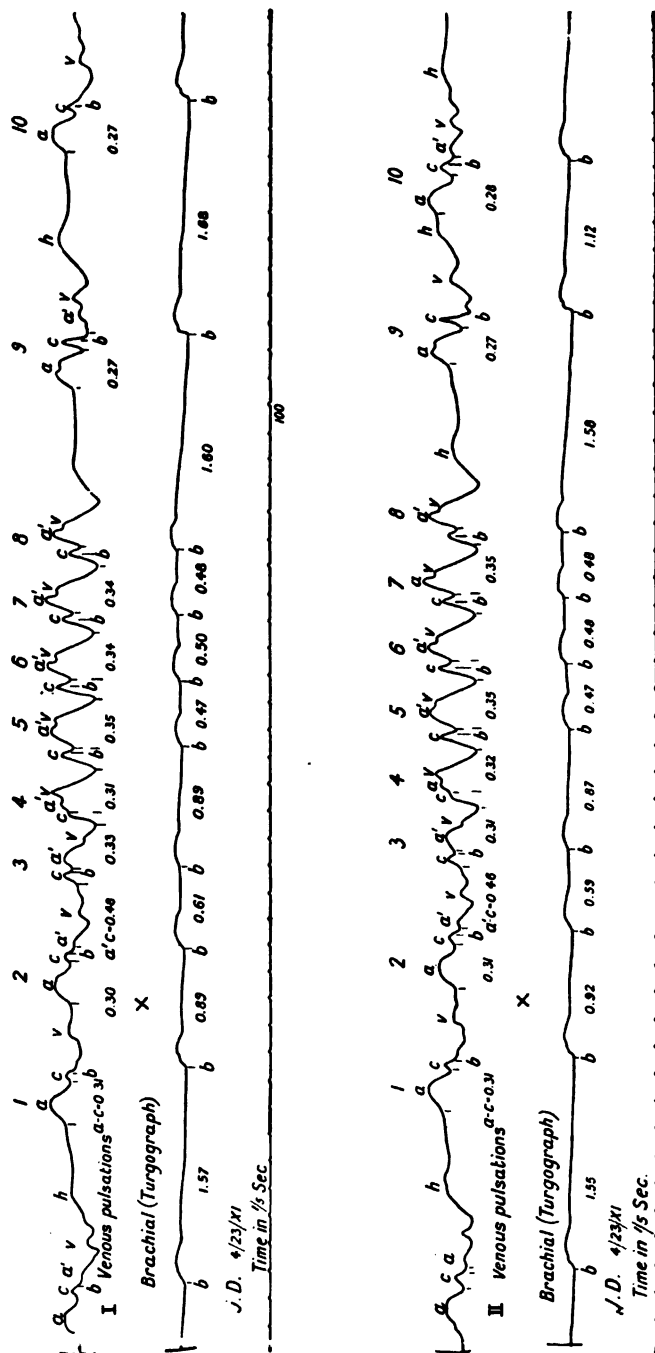


Fig. 3. (Redrawn.) CASE I. Arterial and venous tracings showing two consecutive groups of rapid beats. (April the 23rd, 1911)

characteristic cycles which have been described and a series of four more rapid beats. Here the third cycle of the group produces the usual fused notched wave in the venous tracing, but fails to produce a definite wave in the arterial tracing.

Fig. 4 is an electrocardiographic record which shows a group of rapid beats. The normal electric complex with the usual positive *P*, *R* and *T* waves is seen only in the cardiac cycle number 2 (marked X). In all the others there is distortion in the curve, indicating some cardiac activity just before the onset of the *T* wave, with which an abnormal wave is partly fused. Complexes 3, 4 and 5 represent the three cardiac cycles which always cause characteristic effects in venous tracings, and except for the variations in the relation which the abnormal wave, marked P^1 , bears to the following *R* wave, they do not seem to differ from the other complexes forming the group. This relation of P^1 and *R* will be discussed later. In passing, it is worthy of note that although the third heart-beat of the group never produces as strong an arterial pulse as the others, nothing to differentiate it from the other beats is seen in the size or shape of the *R* wave.

In figure 5 the venous tracing is recorded synchronously with the electrocardiogram, and the relation of one to the other is shown. Two pairs of coupled beats are seen, the first of each pair being a normal beat, showing the usual waves both in the electrocardiogram and in the venous tracing. In the second beat of each couple, the *T* wave in the electrocardiogram is deformed just as are all the *T* waves which occur in the latter part of the curve where a group of rapid beats begins. In these parts of the venous tracing which correspond to the same portions of the cardiac cycle, there is an abnormal wave or distortion, marked a^1 , scarcely noticeable in the first part of the curve, but quite prominent in the latter part.

DISCUSSION OF CASE I.

The cardiac activity which causes these unusual forms of electrocardiographic and venous tracings allows but one interpretation. It is evident that an auricular contraction occurs during ventricular systole, sending a wave into the veins of the neck between the *c* and *v* waves, and moving the string of the galvanometer between

the *R* and *T* waves. The fact that this wave in the electrocardiogram, marked *P*¹, differs conspicuously in form from the normal *P* wave in this case may be taken as evidence that the auricular activity which gives rise to it is ectopic; that is, it originates outside of the usual region of stimulus formation. It will be spoken of, therefore, as the auricular extrasystole. Other examples, recorded by the string galvanometer, of very similar auricular extrasystoles have been published by Lewis (10) and by Rosenthal (16).

EFFECTS OF THE AURICULAR EXTRASYSTOLE.

The most satisfactory interpretation of the various rhythms which this case presents is based on the variety of effects which the auricular extrasystole produces upon the cardiac action. The effect produced depends on whether the auricular extrasystole stimulates ventricular contraction or whether it is blocked.

(a) *Blocked Extrasystoles.*—During the periods of slow cardiac action, the extrasystole never stimulates the ventricles to contract. It may occur during each ventricular systole, as is seen in Fig. 6, when the pulse is very slow and regular. More often, however, every other ventricular systole is accompanied by a blocked auricular systole, as in Fig. 5, and then the slow coupled rhythm takes place. The coupled rhythm is caused by the prolongation of the diastoles following the auricular extrasystoles.

(b) *Effect of the Extrasystoles When They Are not Blocked.*
(1) *Rhythmic success and failure of extrasystoles to control the movements of the ventricles.*—The fact that the auricular extrasystoles may suddenly cease to be blocked and temporarily control the movements of the ventricles is the essential phenomenon in the mechanism of the production of the groups of rapid beats. It is seen in all the curves that show these groups (especially well in Fig. 4) that after a normal cardiac cycle, the group is always inaugurated by an auricular extrasystole which, after an abnormal delay in conduction, stimulates a ventricular systole. There is no evidence of normal auricular activity between this auricular extrasystole and the next ventricular contraction. After the extrasystole

once succeeds in stimulating the ventricles to contract, the stimulus seems to pass more readily from the auricles to ventricles, and the conduction time of the second beat of a group is always much shorter than that of the first. Thus in Fig. 5 the conduction time of the first beat of the group is 0.41 sec., and of the second beat 0.25 sec. This improvement in conduction is only temporary and the time between auricular and ventricular activity gradually lengthens, beat by beat, until the extrasystole fails again to excite ventricular contractions. The slow rhythm is then re-established, the ventricular systoles being preceded by normal auricular contractions, and continues for several beats until the abnormal pace-maker again establishes itself. An exact repetition of the previous group, as seen in Fig. 3, then takes place. It is this alternating success and failure of the auricular extrasystoles to stimulate ventricular contractions that produces the rhythmic changes in the heart-beat.

(2) *The dominance of an extrasystolic rhythm over prolonged periods.*—The auricular extrasystole may, after becoming established as the pace-maker for the ventricles, retain this function over relatively long periods of time. The persistence of the rapid rate is seen in Fig. 7, where it follows suddenly upon a long stretch of coupled beats. The behaviour of the pulse at the time of this change is the same as that which occurs at the onset of a small group of rapid beats. In Fig. 8, synchronous venous pulse tracing and electrocardiogram also show the heart beating at a persistently rapid pace, as in the latter part of Fig. 7. It is seen that the *T* wave of the electrocardiogram is deformed in each cycle by an unusually sharp upstroke, that in the venous tracing there is a large wave between the *c* and *v* waves, and that there is no other evidence of auricular activity. These waves make it certain that an auricular contraction occurs during each ventricular systole, and stimulates the next ventricular systole after a long conduction time. It is clear then that the auricular extrasystole may retain the function of the pace-maker of the heart over a relatively long period of time, and produce a rapid and regular heart action.

THE ACTION OF THE CARDIAC NERVES.

(a) *Effect of Psychic Excitement.*—A number of observations were made which seem to indicate a distinctly abnormal nervous control of the heart in this case. It was found that under psychic excitement, the heart could beat with a practically normal rhythm, entirely undisturbed by auricular extrasystoles. In April, 1910, the patient was brought from Philadelphia to New York in order to obtain electrocardiograms, through the kindness of Dr. Walter B. James. It was noticed during the journey that the arrhythmia had disappeared and the heart was beating practically regularly at about 80 per minute. The pulse continued to be regular during the journey and after arriving at the Presbyterian Hospital, where exercise, rest, ice to the præcordium and other measures failed to disturb the normal rhythm. At this time the curve shown in Fig. 9 was obtained. The electrocardiogram is quite normal as regards the form of the various waves, although the conduction time is abnormally long. It measures 0.20 to 0.21 sec., but is shorter in this curve than in those obtained during the usual abnormal rhythm. This curve shows a definite irregularity in the lengths of the individual cycles, a conspicuous sinus arrhythmia being present.

The heart continued to beat in a practically normal manner for three days. The usual arrhythmia appeared only once during this time, and then for a short period only, after the patient had been resting quietly for some time, but the heart resumed the normal rhythm before records could be obtained. Finally, on the morning of the fourth day, a persistent arrhythmia established itself and records of groups of rapid beats resembling those in Fig. 4 were made. During the years of observation, this has been the sole occasion upon which persistent normal action has been recorded.

(b) *The Effect of Ice upon the Præcordium.*—On several occasions it was noticed that the cardiac rhythm could be changed by applying ice to the præcordium, the patient being quiet in the recumbent position. This effect was obtained only when the heart was beating at an abnormally rapid rate, as in Fig. 8. The change usually took place in from five to fifteen minutes, and either the usual group rhythm or a slow regular pulse resulted. On one occasion the heart rate was 123 per minute and regular before the

application of ice. About five minutes afterward, the rate had fallen to 42 per minute (Fig. 6). Deep inspiration seemed sometimes also to change the rapid rhythm into a slow regular or coupled rhythm, or into the grouped rhythm.

(c) *The Effect of Vagus Pressure and Atropine.*—Pressure over neither the right nor the left vagus nerve produced any change in the cardiac rhythm, and from this fact it was considered that the vagus tone was low. In order to test this question further, the patient, who was at the time under observation in the Hospital of The Rockefeller Institute, was taken to the electrocardiographic room and a series of records were made. Atropine sulphate (0.6 mg.) was injected hypodermically and about fifteen minutes later the changes in the rhythm, which had been obtainable by very deep forced inspiration, were no longer elicited. The heart increased in rate in a startling manner and beat perfectly regularly at 180 per minute. At this time the patient developed cardiac sensations, which he had never had before and which caused him considerable anxiety. He said he felt as though the heart were trying to leap out of the chest. The cardiac contractions were very violent and shook the whole thorax. A striking feature of the condition at that time was the reference of all symptoms to the violent heart action. There was neither dyspnoea nor any change in colour. All the ventricular beats produced radial pulsations; these, however, were small and soft. In about two hours slight slowing followed deep inspiration, and in about two and a-half hours after the administration of the drug, this effect of deep inspiration was conspicuous. The heart action quieted down after this interval. Later in the day the patient felt quite well and showed no objective signs of cardiac inadequacy. Fig. 10 was obtained while the patient was under the effect of atropine; the rate was 164 per minute.

The experience with atropine throws much light on the nervous control of the heart in this case. It is well known that atropine removes the heart from vagus control by producing a paralysis of the inhibitory nerve endings, and there seems to be no doubt that it was through the removal of the vagus inhibition that the very rapid heart action resulted. As the paralysis of the vagus action caused so great an increase in rate, it must be concluded that the vagus tone

was constantly high and that its inhibitory power controlled a heart which would otherwise have beat at an abnormally rapid rate.

The fact that the patient had marked cardiac sensations only during the height of the rapid rate after atropine suggests the possibility that at this time the heart-beat passed the so-called critical rate described by Wenckebach (21). Symptoms which may arise and disappear suddenly with changes of rate during attacks of paroxysmal tachycardia, have been ascribed with good reason by Wenckebach to what he calls auricular plugging ("Pfropfung"). The symptoms apparently arise when the auricles, contracting during the ventricular systole of the preceding cardiac cycle, are unable to propel the blood forward into the ventricles. He points out the rôle which delay in conduction may play in producing the coincidence of auricular and ventricular systole and shows how it interferes with the pumping mechanism of the heart. Fig. 10 shows this coincidence of auricular and ventricular contraction, for the *P* wave is seen to lie between the *R* and *T* waves. The mechanism which Wenckebach has described is therefore present here, and might account for the symptoms which were observed. In Fig. 8, however, both the electrocardiogram and the venous tracing show the same coincidence of auricular and ventricular systoles that is seen in Fig. 10. Although there is a considerable difference in rate, this auriculo-ventricular relation is maintained by the lengthened conduction time. It appears then that the mechanical conditions were quite suitable in both instances (Fig. 8 and 10) for the production of symptoms. But since none were present when the curve in Fig. 8 was obtained, some doubt is attached to the mere coincidence of the auricular and ventricular systole as the sole factor in the production of symptoms after the heart has passed the so-called critical rate in paroxysmal tachycardia.

THE PRODUCTION OF THE AURICULAR EXTRASYSTOLE.

The auricular extrasystole presents some features which must be considered with the hope of gaining a clearer insight into the various phenomena of this case. It is a striking fact that the extrasystole never occurs except when preceded by a ventricular systole. Both

the electrocardiograms and the graphic records show that under all conditions the onset of the auricular extrasystole occurs at a constant length of time after the onset of ventricular systole. This time is so short that the extrasystole begins before the end of ventricular systole, always accompanying rather than following the ventricular activity. The extrasystoles in the curves of Lewis and of Rosenthal, mentioned above, also showed a constant relation between their onset and that of the preceding ventricular systoles. This constant relation suggests that the ventricular activity in some way determines the onset of the auricular extrasystole, or that both are determined by the same factor. If curves such as those in Fig. 8 and 10, when a constantly rapid rate is maintained, were studied alone, the conclusion might seem justified that some point in or near the atrio-ventricular system had assumed the function of the cardiac pace-maker. But when the other curves are considered, this assumption is very improbable. When the rate is slow these extrasystoles occur during ventricular contractions which are obviously inaugurated by the preceding normal auricular systoles. It is unnecessary, therefore, to consider that the junctional tissues are the site of common stimulus production for the sequential ventricular systoles, and for the auricular extrasystoles. The point of origin of the extrasystoles must be left undetermined. When the rate is rapid, the time relations between the onset of the auricular extrasystoles and of the ventricular systoles are such that it seems impossible that both auricles and ventricles could be stimulated from some abnormal point lying between them. The *P-Q* time (Fig. 10) is 0.18 to 0.20 sec., while the *Q-P* time is about the same. If some point in the junctional tissues stimulated both parts of the heart simultaneously, these points, indicating the onset of auricular and ventricular contractions, would fall nearer together. The mechanism of the heart-beat when the rate is rapid is therefore not dependent on atrio-ventricular stimuli. The origin of the extrasystoles, both when it is followed by ventricular systoles and also when it is blocked, lies probably above the junctional tissues. The significance of the constant relationship between the onset of ventricular contractions and of the auricular extrasystoles is not clear.

THE RÔLE OF THE CARDIAC NERVES IN DETERMINING THE VARIATIONS IN RHYTHM.

This case presents a variety of rhythms which change from one to another readily and suddenly. Psychic excitement, ice to the præcordium, rest and the administration of atropine are factors which all influence the cardiac rhythm. It is evident, therefore, that extracardiac influences can change the rhythm, and that these influences are apparently active through the nervous mechanism controlling the heart. Besides the variations in the effect which the auricular extrasystoles have on the ventricular rate, there are also variations in the occurrence of the extrasystoles, as at times they occur with every ventricular systole, at times with every alternate systole and during one period they failed to occur.

There is comparatively little experimental evidence for the belief that such abnormal cardiac rhythms are produced through the action of the cardiac nerves. It is well known that the so-called cardiac properties of rhythmicity, conductivity, irritability and contractility are under the influence of the vagi, which nerves are in a state of constant tone. Hunt (5) has shown that the cardiac accelerators are also in tonic activity and that the most important functions of the accelerators seem to be connected with their constant state of tone. Hunt's work and that of Rothberger and Winterberg (17, 18 and 19) lead to the conception that the cardiac functions stand between a constantly balanced nervous mechanism, the vagi opposing the accelerators. The state of the various cardiac properties at any one time will depend then on the relative predominance of the vagus influence over the accelerators, or *vice versa*. Hunt's experiments led him to conclude that change in vagus tone was the most important factor in the production of changes in the heart-beat in the normal mammal.

In the case under discussion there is at least one fact which demonstrates that the nervous control of the heart was abnormal and suggests that nervous influences were largely responsible for the variations that were seen. When atropine was given, the heart action was very rapid and the conduction time was reduced to within the normal limit. At this time it can be safely asserted that the action of the accelerator nerves predominated over the action of

the paralysed vagi. By analogy it seems very probable that the tone of the vagi was reduced when a constantly rapid rhythm, as seen in Fig. 8, was in progress. It seems probable also that the changes from this type of rapid rate to slower rates when the auricular extrasystoles became blocked, resulted from an increase of vagus tone relative to that of the accelerators. The rhythmic changes of the heart-beat may be explained, therefore, by rhythmic variations in the nervous control of the heart. When the accelerator tone was relatively high, the extrasystoles accompanied all ventricular systoles and were always able to stimulate ventricular contractions. When the vagus tone was relatively high the extrasystoles occurred with alternate ventricular systoles only, and were always blocked. During the times when these two conditions alternated, causing the characteristic rhythmic changes, it may be assumed that the tone of the vagi was periodically increased and diminished relatively to that of the accelerators. During the period of several days when the auricular extrasystoles were absent and the heart action was relatively normal, the condition of the heart seemed to be farther removed from its condition after atropine than at any other time. It would seem likely, therefore, that at that time the highest degree of vagus tone was present and that psychic excitement was the cause of raising the vagus tone.

Although periodic changes in the tone of the cardiac nervous mechanism may account for the rhythmic changes in the heart-beat, the fact that the changes occurred so often each time after the same number of beats suggests that rest and fatigue of the heart muscle may itself play a part in the determination of the rhythmicity. The heart muscle, however, would be held by the cardiac nerves in such a state that neither the rapid nor the slow rate could be permanently maintained, so that they probably do play a part at all times in determining the cardiac action, although under certain conditions rhythmic rest and fatigue of the muscle itself may come in as a causative factor.

SUMMARY.

A variety of disturbances of cardiac rhythm have been described in an otherwise healthy boy. The usual type of disturbance in this

case was one in which the rate altered in a rhythmic manner, producing small groups of rapid beats between which slow and regular or coupled beats occur. Either the rapid rate of the groups or the slow rate occurring between them might persist over a relatively long period of time. These various changes in the cardiac rhythm were the result of the effects which auricular extrasystoles have on the heart-beat. Different effects were produced, according as the extrasystoles stimulated ventricular contractions or were blocked. The alternate success or failure of the auricular extrasystole in stimulating the ventricles caused the rhythmic changes in the heart-beat.

The auricular extrasystoles bore in all types of rhythm, a constant relation to the preceding ventricular systole. The significance of such a constant relationship is not clear.

The nervous control of the heart was distinctly abnormal and the cardiac rhythm was very susceptible to various agents which influenced the cardiac nervous mechanism. Psychic excitement was accompanied by a normal heart-beat, and this was seen under no other conditions; atropine caused a rapid tachycardia. The changes in the cardiac rhythm were apparently the result of changes in the nervous control of the heart, although the frequent and rhythmic variations may have depended in part on alternating states of rest and fatigue of the heart muscle itself.

PART II. (CASE 2.)

History and Physical Examination.—The second case of rhythmic changes in the heart-beat occurred in a girl of seven, who suffered from mitral insufficiency, apparently of rheumatic origin, and probably from myocardial insufficiency. She was admitted to the Hospital of the Rockefeller Institute on April the 18th, 1911, in a state of fair compensation. Besides tonsilitis before the age of five and diphtheria at that age, the patient had an attack of acute articular rheumatism two months before admission. Her cardiac symptoms, consisting largely of dyspnoea, usually had occurred only after exercise. On several occasions definite breaks in compensation followed violent exertion. On admission the physical examination

was practically negative except that the heart was enlarged. The area of cardiac dullness extended 4.25 cm. to the right and 12.5 cm. to the left. There was a systolic murmur of mitral insufficiency at the apex and the heart was beating forcibly 110 times per minute.

Digitalis was administered (0.5 c.c. of the tincture every four hours) from April the 29th until May the 7th. On April the 30th it was first noticed that an irregularity simulating a sinus arrhythmia was present, and it seemed even more noticeable on the following day. The arrhythmia persisted and on May the 8th it consisted of a series of fairly rapid beats, then two slower cycles, and then another series of rapid ones. Conspicuous slowing followed deep inspiration. On the next day it consisted of two, or occasionally three slow irregular cycles, followed by four, five or six rapid ones. Deep inspiration caused a considerable increase in the number of slow irregular beats. The patient left the hospital on this day in good condition and returned for observation three days later, when the heart was beating regularly. The arrhythmia was not present when the patient was seen again in August, 1911.

Description of the Curves.—Records of the arrhythmia were obtained in synchronous tracings from the venous and brachial pulse

TABLE I.

Length of Cardiac Cycles in Seconds.

Measured from the brachial tracing in Fig. 11 and in the remainder of the curve.

CYCLES OF THE SLOW RHYTHM.			CYCLES OF THE RAPID RHYTHM.				
1st	2nd	3rd	1st	2nd	3rd	4th	5th
0.89	0.94	0.80	0.59	0.56	0.56	—	—
0.88	0.92	0.82	0.60	0.60	0.62	—	—
0.90	0.93	0.85	*	*	*	—	—
*	0.89	0.83	0.61	0.58	0.57	0.61	0.67
0.92	0.96	0.85	0.61	0.57	0.58	0.61	—
0.91	0.93	0.88	0.60	0.56	0.59	0.63	0.65
0.91	0.95	0.81	0.61	0.57	0.55	—	—
Average 0.90	0.93	0.83	0.60	0.57	0.58	0.62	0.66
Average Rate 67 per Minute.			Average Rate 102 per Minute.				

* Cycles occurring in part of tracing that was missing.

and in electrocardiograms. A comparatively long graphic record was made, part of which, reproduced in Fig. 11, is characteristic of

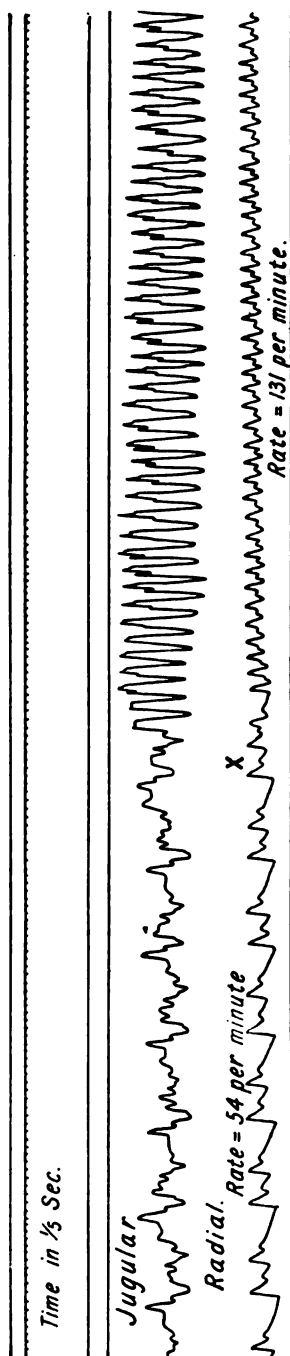


Fig. 7. (Redrawn.) CASE 1. Arterial and venous tracings showing a relatively persistent bigeminal pulse, changing into a relatively persistent rapid pulse.

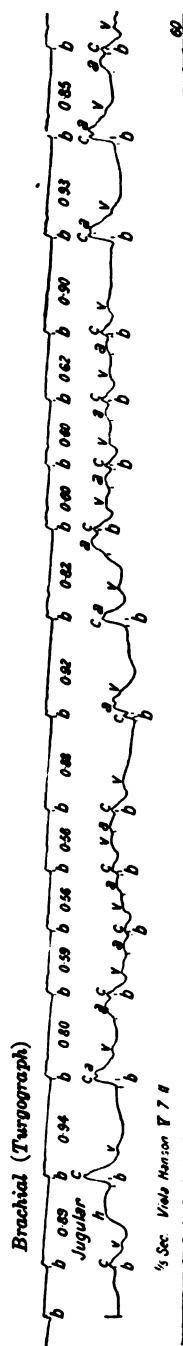


Fig. 11. (Redrawn.) CASE 2. Arterial and venous tracings showing three groups of slow beats, alternating with rapid beats. Measurements are in seconds.

the entire curve and shows three groups in each of which there are three slow and three rapid beats. Seven groups of slow beats occurred in the entire tracing, and either three, four or five rapid beats occurred between them. The measurements of the lengths of all the cardiac cycles, as measured from the brachial tracing, are tabulated. From the table it is seen that the remarkable duplication of the groups is not confined only to their regular recurrence, but is noticeable also in the lengths of the various corresponding cycles, especially in the slow beats. The second beat of each group is longer than the others, while the third is the shortest, and the length of the corresponding beat in each group is strikingly constant. In the group of rapid beats, the second beat is usually a little shorter than the first, and a tendency for the beats to lengthen, as the rapid group progresses, is seen, especially when the rapid group is composed of four or five cycles. These details indicate the definite rhythmicity in the changes in rate which occurred.

The jugular tracing in Fig. 11 shows three pairs of large unusual waves which accompany in each instance the last two of each group of slow beats. Between these pairs of waves, four cardiac cycles are seen, each represented by the usual *a*, *c* and *v* waves. These two series of normal waves have a very close resemblance to each other. The points in the venous tracing which are synchronous with the onset of the brachial pulse (marked *b*) always fall not only just after the onset of the normal *c* waves, as is to be expected, but also after the onset of the large unusual waves as well, at about the same length of time. As there is no evidence of auricular activity before these waves, it seems clear that the onset of these large waves represents the *c* wave, and that they are produced by ventricular activity. There are prominent waves following closely and partially fused with the *c* waves which undoubtedly represent auricular activity. The occurrence then of the waves in the venous tracing which are caused by auricular contractions, marked *a*, is not regular, and it is evident that after every four or five normal cardiac cycles, conspicuous slowing of the auricular rate takes place. The slow auricular rate continues for only two beats. During these two beats the ventricular systoles do not

follow but precede the auricular systoles, the *c* waves preceding the *a* waves in the venous tracing.

The electrocardiogram (Fig. 12) shows a group of two slow auricular intervals (*P-P* time), followed by seven rapid beats. The relation of the *R* waves to the *P* waves during the auricular slowing is abnormal. The onset of the *R* wave is abnormally close to the onset of the *P* wave, sometimes practically coinciding with it. This relation makes it evident that the ventricular contractions during the slow beats are not dependent for their stimuli upon the auricular activity.

DISCUSSION OF THE CURVES.

These curves show two abnormal factors which together determine the form of the rhythmic change in the cardiac activity. The first is a periodic change of rate of the auricular contractions. The second is the occurrence of an abnormal relation of the auricular and ventricular contractions during the periods of slow auricular activity. Each factor needs to be discussed separately.

(1) *The rhythmic change in the auricular rate* is associated in this case apparently with the administration of digitalis, as it occurred only after the patient was well under the effect of the drug, and disappeared several days after the drug was stopped. The tracing of the respiratory movements, taken synchronously with the electrocardiogram, shows that the sudden slowing of the auricular rate bears no relation to respiration. This observation is in accord with that of Turnbull (20) who observed marked changes in rate of the whole heart, independent of the respiratory movements, following the use of squills, which he considered as dependent on changes in vagus tone. There was also in his case considerable depression of conductivity leading to partial heart-block.

The rhythmic changes of rate which occurred in our case seem very probably to have resulted from the action of the drug on the heart through the vagi. It is well known that digitalis acts on the heart, at least in part, through its ability to raise the vagus tone, but usually the increase of vagus action has a more marked inhibitory effect on the conductivity than on the rhythmicity of the heart. There is, in fact, considerable evidence for the belief, espe-

cially emphasized by Cushny (2), that the therapeutic value of digitalis depends largely upon its ability to lower conductivity. In this case conductivity was not lowered, as the *P-R* interval in the electrocardiogram never exceeds 0.17 sec., which is within the normal limits. The drug has then apparently produced a disturbance in rhythmicity without affecting conductivity.

(2) *The abnormal relation of the auricular and ventricular contractions* during the periods of slow auricular rate is seen in both the venous tracings and in the electrocardiograms. As has been pointed out, during these periods the ventricular contractions are not dependent for their stimuli upon the auricular contractions. It is clear also that the auricular and ventricular systoles in the slow cardiac cycles are not the result of fixed ectopic stimulus formation common to both, as it is indicated in both the venous tracings and the electrocardiograms that the relation of these activities to each other is not constant. The fact also that the *P* wave of the electrocardiogram in these abnormal cycles is not deformed shows that the course of the contraction through the auricle is normal. The ventricular complexes are also not deformed. It is clear, therefore, that the impulses for their contractions arise in some point between the auricles and the ventricles and pass to the latter in a normal manner. The curves indicate, therefore, that the stimulus formation for the ventricles is taken up by some independent point which possesses an inherent rate of rhythmicity higher than that of the auricles during their slow periods, and ventricular contractions result.

The inherent rate of the point which becomes the pace-maker of the ventricles when they beat independent of the auricles, is in this instance about sixty beats per minute. The action of digitalis had no effect on the inherent rate of this point, as ventricular contractions at the same rate appeared when the patient was free from the effect of the drug and when the auricular rate was slowed by pressure on the right vagus nerve. The curves showing this result of vagus pressure in this case were published in a previous communication (15) (Fig. 12-15). Although nothing is known as to the rate at which the ventricles of a normal child of seven would beat independently of the auricles, it seems very likely, from what

is known in general of independent ventricular rhythmicity, that this rate of sixty beats per minute is abnormally high. An even higher inherent ventricular rate has been encountered in two other cases of endocarditis in children, in which the ventricles beat spontaneously whenever the auricular rate was sufficiently slowed by stimulation of the right vagus nerve. These cases have been discussed in a previous paper (15). The curves showing this dissociation resemble in form those obtained by Rothberger and Winterberg (17) during right vagal and left accelerator stimulation in dogs. In their experiments the vagus stimulation lowered the rate of the heart-beat as set by the sino-auricular node, while the accelerator stimulation raised the inherent rate of the ventricles, thus causing it to exceed that of the slowed auricles. In our cases the right vagus alone was stimulated, and we think that the ventricles established their independent rhythm because their inherent rate was raised by a constant abnormal activity of the accelerator nerves, or through a heightened susceptibility of the heart muscle to the action of the accelerators.

SUMMARY.

The curves from a child of seven, suffering from chronic endocarditis, showed rhythmic changes in the heart-beat which were determined by two abnormal factors. First, there was a sudden and profound change in the auricular rate, occurring in a definitely rhythmic manner. This was apparently an effect of digitalis. Second, the ventricles beat independently of the auricles during the periods of slow auricular rate. This occurred because the inherent rate of the site of ventricular stimulus formation exceeded that of the retarded auricles. The actual rate was high, and was occasioned, we believe, by a hypertonus of the cardiac accelerator nerves or of a heightened susceptibility of the heart to the action of these nerves.

PART III. (CASE 3.)

History.—A physician, aged fifty-six, who lives in a high altitude and who is an active man, noticed about the year 1900 that he had occasional cardiac irregularity. This irregularity consisted of an

intermission, occurring after a sequence of six or seven beats. At first, withholding tobacco for a few days seemed to induce more regular heart action. A trip towards sea level had the same effect. For a long period mental diversion, alcoholic stimulation or excitement seemed to bring about at once a slower and regular rhythm. The irregularity had become steadily more pronounced and when he was seen was not so amenable as formerly. Except for the discomfort of the sensations of cardiac irregularity, no serious inconvenience has been suffered. The patient had dizzy spells and tinnitus aurium at times for many years, and on several occasions fainting attacks have been narrowly averted. Stabbing heart pains have occasionally been annoying. Tobacco had not been used immoderately, and he had taken very little alcohol.

The patient had scarlet fever at the age of ten and pneumonia at sixteen. He was very athletic in college between the ages of seventeen and twenty-one, and then turned suddenly to the sedentary life of a laboratory worker. About ten years after graduation, he developed pulmonary tuberculosis, from which he entirely recovered after five or six years. He had typhoid fever in 1896 after which he had a "typhoid rib," for which he was operated on (curetted). Healing required eighteen months.

Physical Examination.—Physical examination on June the 16th, 1911, showed practically nothing abnormal. When the patient sat, percussion demonstrated that the right cardiac border coincided with the left sternal margin. The superior outline of relative dulness lay in the third left interspace, and ran down 2.2 cm. to the left of the left nipple to the sixth interspace, 12.5 cm. to the left of the mid-sternum. The apex beat was under the sixth rib and in the sixth space, and its outer border being 12.5 cm. to the left of the mid-sternum. The first heart sound at the apex was impure and "scrapy," and the interval between the first and second sounds was abnormally short. The other sounds were clear. The pulse rate whilst sitting was 116 per minute, not counting frequent intermissions. The blood pressure (Janeway instrument) was 110 mm. mercury for the strong beats which came through the cuff after the pauses; the weaker beats forming the main part of the rhythm

came through at about 104 mm. The urine has always been normal.

Description of Curves.—The electrocardiograms (Fig. 13 and 14) were obtained after the patient had been smoking, and show groups of rapid beats divided from each other in a rhythmic manner by single prolonged cardiac cycles. The two curves differ from each other because the first was obtained with the second lead, the right arm and left leg, while the second was obtained by the third lead, the left arm and left leg. They differ also in that there are six beats (Fig. 14) in a group in one tracing and seven beats (Fig. 13) in a group in the other. In Fig. 13 (second lead), after a long diastole, a cycle occurs which is represented by a well defined positive *P* wave, a very small *R* wave, a well marked *S* wave and a low, rather elongated *T* wave. The unusual *R* wave is constant throughout the curve and its form need not concern us here. The *T* wave is apparently unchanged except by being deformed through partial coincidence with the wave that follows it. The important and striking change occurs in the *P* wave, which is reversed or negative in all the cycles which follow until the next pause. This change in the shape of the *P* wave indicates that the stimulus of contraction arises in some abnormal point and passes through the cardiac structures in an abnormal fashion. In other words, the negative *P* wave indicates that it represents an ectopic auricular systole which by its rhythmic occurrence controls the heart rate.

A study of this curve reveals the following facts: After a pause of the heart in diastole, a cardiac cycle of normal sequence occurs. The diastole following this normal beat is short and is interrupted by an abnormal auricular systole, which stimulates a ventricular systole after a normal conduction time. Stimuli continue to arise at the abnormal point at a fairly rapid rate for five more beats, each followed by a ventricular contraction. Then a period occurs in which stimuli cease to be generated in the abnormal point, and a pause again occurs which allows the normal cardiac pace-maker to re-establish itself. The normal mechanism is dominant for one beat only, when the ectopic impulses again resume control.

The same mechanism is shown in Fig. 14, except that the pause

occurs after five rapid beats instead of after six. The lengths of the cardiac cycles, as measured from the beginning of one ventricular systole to the next, vary considerably, and fall into three groups. The long cardiac cycles which include the pauses average 1.03 seconds in length and indicate a heart rate of 58 beats a minute. The first cycles after the pauses average 0.55 seconds in length and indicate a rate of 109 beats a minute, while the remaining beats of the groups average 0.45 seconds in length, indicating a rate of 133 beats per minute. Besides the rhythmic occurrence of beats belonging to these three groups, the beats of each group have a tendency to shorten as the end of the group is approached. As all these beats arise from the same abnormal point of stimulus formation, it is evident that the rate of stimulus formation gradually increases until for some reason the abnormal stimuli suddenly cease. This sudden cessation suggests that fatigue sets in and that rest is necessary before the function of stimulus formation can be resumed. It is probable, therefore, that a rhythmic alteration in the excitability occurs in some auricular region which is capable of generating stimuli, and that the change in excitability is determined by periods of rest and fatigue of the heart muscle itself.

SUMMARY.

The curves from this healthy man of fifty-six show rhythmic changes in the heart-beat which are caused by the fact that an abnormal auricular region generates stimuli at a rate faster than that of normal stimulus formation. The region therefore becomes the cardiac pace-maker, a function which it seems unable to maintain constantly. After a series of five or six abnormally rapid beats, this ectopic pace-maker fails to produce stimuli and a pause in its activity occurs, during which one normal cardiac cycle is developed. During this pause rest allows the abnormal pace-maker to resume its stimulus-forming function which ceases again after the same number of beats as before, on account of fatigue of the heart muscle itself.

GENERAL DISCUSSION.

In the three cases that have been described, the feature common to all is the rhythmically recurring changes in the heart-beat. Such

striking phasic changes are apparently rare. There is one form of rhythmic change in the rate of the ventricular contractions, however, which is not very infrequent and which occurs in partial heart-block. It is produced when every third or fourth auricular systole fails to stimulate a ventricular systole and when the conduction time in a group of beats shows progressive lengthening until the block occurs. Partial heart-block due to digitalis frequently exhibits this condition.

Rhythmic changes in the heart-beat were first experimentally investigated by Luciani (12), who studied the periodic action of the suspended frog's heart under numerous conditions. Some of the curves show fairly definite rhythmic changes. Among others, Öhrwall (13) and later Langendorff (6) have discussed these peculiarities of heart action which are associated especially with certain stages of partial cardiac asphyxiation. Öhrwall considered that changes in the heart muscle, especially in excitability, underlay the rhythmic changes, while Langendorff thought that changes in the controlling nervous mechanism were probably primarily responsible. The cardiac action of the cases here described is quite different from the so-called Luciani periods. We have recently obtained electrocardiograms of what seem to be true Luciani periods in the human heart from dying patients, and they show groups of beats of the whole heart, followed by pauses sometimes a full minute in length. These observations have been described in detail in another communication (14).

Wenckebach (22) has drawn an analogy between the cardiac rhythm observed in a patient and the Luciani periods. His case resembles superficially our first case. The details of the venous curves are not entirely clear, owing to the rapidity of the cardiac rate, but it is almost certain that the periodic changes of rate affected the whole heart and auricular extrasystoles played no part. In this respect his case does resemble more closely the Luciani periods than do ours. Wenckebach interprets the periodic changes in rate as caused by depression of excitability and rhythmicity of the heart through the activity of the cardiac nervous mechanism. It is an example of rhythmic changes in the heart-beat which is different from any we have observed.

A case resembling closely our second case has been described by Lewis (11). The patient was under the influence of digitalis and the rhythmic changes of rate seemed to result from its action through the vagi. Not only rhythmicity but also conductivity was disturbed, however. With the slow auricular rate the ventricles inaugurated their own contractions, just as occurred in our case. Belski (1) has also published a curve (Fig. 9) resembling the polygraphic tracings from our second case. Except for rhythmic changes produced by regularly recurring extrasystoles, such as that reported by Laslett (7), no other forms of rhythmic changes in the human heart-beat have been described, so far as we know.

THE RELATION OF OUR CASES TO OTHER FORMS OF ARRHYTHMIA.

The classification of the cardiac arrhythmias in general use, that advocated by Hering (4), offers no satisfactory place for these now described, although extrasystoles, auricular in the first and third and ventricular in the second, play a rôle in all. They cannot be considered, however, as extrasystolic arrhythmias in the usual sense.

A more interesting consideration is the relation which the first and third cases have to paroxysmal tachycardia. Largely through Lewis' (8, 9 and 10) work it now seems clear that the paroxysms of tachycardia occur when some region of the heart outside of the sino-auricular node takes over the function of impulse formation. This ectopic impulse formation becomes apparent only when the impulses are generated at a rate higher than that of the sino-auricular node, for only then does the abnormal region become the cardiac pace-maker.

In the first and third cases presented in this paper, a rapid cardiac rhythm occurs when a site originating ectopic auricular systoles stimulates the ventricular contractions. An abnormal activity of the accelerator nerves may here play a part in determining the ectopic auricular activity which becomes the cardiac pace-maker for short, interrupted periods. There is in one of these cases some evidence that the accelerators were abnormally active. There is also experimental evidence that the rate of stimulus formation may

be raised by accelerator stimulation in abnormal regions above that of the sino-auricular node. Thus Hering (4) has found that accelerator stimulation may not only quicken the heart-beat, but may produce tachycardia. Rothberger and Winterberg have shown that accelerator stimulation raises the rate of rhythmicity of points in the ventricles of hearts poisoned by barium and calcium above that of the sino-auricular node, thus producing ventricular tachycardia. Possibly the normal pace-making region works usually relatively near its maximum rate, while other regions may be raised by influences outside of the heart itself, especially by stimulation of the accelerator nerves, to a much higher rate of rhythmicity than is normally present. Although the mechanism underlying each of these two cases may be the same as that underlying paroxysmal tachycardia, the rate of the rapid beats is not so high as usual, nor is it constantly maintained.

GENERAL SUMMARY.

In the three cases that are described, changes in the heart rate have been observed by graphic records which occur in a rhythmic manner. The phasic character of these changes is the only feature common to all. In the first case the rhythmic change was the result of the alternating success and failure of ectopic auricular systoles to stimulate ventricular contractions. In the second case the auricular rate changed rhythmically and became periodically so slow that the ventricles were able to inaugurate their own rhythm. The periodic changes in auricular rate resulted from the administration of digitalis. In the third case ectopic auricular systoles occurred in groups at a rate more rapid than that of the normal sino-auricular rhythm, and the site from which they arose became periodically the cardiac pace-maker.

The two cases in which ectopic auricular systoles periodically became the cardiac pace-maker are apparently closely allied to or identical with paroxysmal tachycardia so far as the mechanism underlying the abnormal cardiac action was concerned.

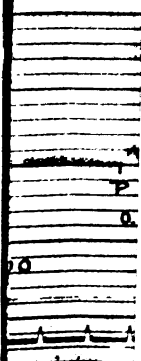
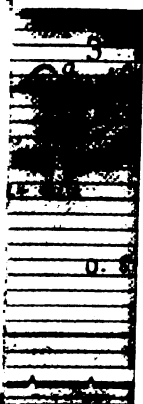
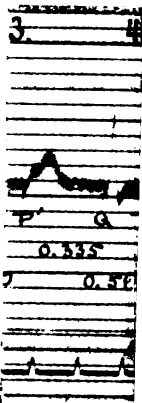
In the first two cases the nervous mechanism controlling the heart-beat seemed to be abnormal and played an important but not an

exclusive rôle in the production of the rhythmic changes. In the third case, although there is less evidence of such an abnormality the sudden appearance of the unusual cardiac activity suggests that here also the nervous controlling mechanism was a factor in determining its onset.

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